FACULTY OF VETERINARY MEDICINE
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DNA ADDUCT FORMATION DUE TO THE GASTROINTESTINAL DIGESTION OF RED MEAT

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2D-LC	Two-Dimensional LC
A	Adenine
AαC	2-amino-9H-pyrido[2-3-b]indole
AFB_1	Aflatoxin B_1
AMS	Accelerator Mass Spectrometry
ANOVA	Analysis of variance
ATNC(s)	Apparent Total N-nitroso Compound(s)
BHI	Brain Heart Infusion (broth)
BTX-2	Brevetoxin B
C	Cytosine
$CaCO_3$	Calcium carbonate
CNL	Constant Neutral Loss
COX-2	Cyclo-oxygenase 2
CRC	Colorectal Cancer
CRO	Crotonaldehyde
CrodG	α-methyl-γ-hydroxy-1,N_2-propano-2'-deoxyguanosine, the main CRO adduct with deoxyguanosine
CroG	α-methyl-γ-hydroxy-1,N_2-propanoguanine, the main CRO adduct with Guanine
CT-DNA	Calf Thymus DNA
DD-LC-MS	Data-Dependent LC-MS
dG	Deoxyguanosine
DiMeIQx	2-amino-3,4,8-trimethyl-3H-imidazo[4,5-f]quinoxaline
DNA	DeoxyriboNucleic Acid
EMA	European Medicines Agency
ESI	Electrospray Ionisation
EtDA	Ethyldiazoacetate
Fapy-A	Formamidopyrimidine-Adenine
Fapy-G	Formamidopyrimidine-Guanine
FDA	US Food and Drug Administration

FWHM	Full Width Half Maximum
G	Guanine
GC	Gas Chromatography
GC-ECD	Gas Chromatography – Electron Capture Detection
GIT	Gastrointestinal tract
GluP1	2-amino-6-methyldipyrido[1,2-a:3,2-d]imidazole
H_2O_2	Hydrogen Peroxide
H_2S	Hydrogen Sulfide
HCAs	Heterocyclic Amines
HCl	Hydrogen Chloride
Hep-G	Heptenal-Guanine
Hex-G	Hexenal-Guanine
HESI	Heated ElectroSpray Ionisation
HFBe	High fat beef diet
HFCh	High fat chicken diet
HNE-G	Hydroxynonenal-Guanine
HO·	Hydroxyl radical
HPLC-ECD	High Performance LC - Electron Capture Detection
HPLC-FD	High Performance LC – Fluorescence Detection
HRAM	High Resolution Accurate Mass
HRMS	High Resolution Mass Spectrometry
IARC	International Agency for Research on Cancer
IQ	2-amino-3-methylimidazo[4,5-f]quinolone
IQx	2-amino-3-methyl-imidazo[4,5-f]quinoxaline
KDA	Potassium diazoacetate
LC	Liquid Chromatography
LFBe	Low fat beef diet
LFCh	Low fat chicken diet
LM-PCR	Ligation-Mediated - Polymerase Chain Reaction
LOD(s)	Limit(s) of Detection
LOQ(s)	Limit(s) of Quantification
LPO(s)	Lipid Peroxidation Product(s)
M_1A	Oxopropenyl-Adenine; Malondialdehyde-x1-Adenine

M_1AA	M_1-acetaldehyde-A; adduct of 1 malondialdehyde and acetaldehyde molecule with Adenine
M_1C	Oxopropenyl-Cytosine; Malondialdehyde-x1-Cytosine
M_1dG	3-(pyrimido[1,2-α]purin-10(3H)-one; Malondialdehyde-x1-Guanosine
M_1G	Pyrimido[1,2-a]purin-10(1H)-one; Malondialdehyde-x1-Guanine
M_2AA	M_2-acetaldehyde-A; adduct of 2 malondialdehyde and 1 acetaldehyde molecule(s) with Adenine
M_2-acetaldehyde-G	Adduct of 2 malondialdehyde and 1 acetaldehyde molecule(s) with Guanine
M_2G	Adduct of 2 malondialdehyde molecules with Guanine: Malondialdehyde-2x-Guanine
M_3C	Adduct of 3 malondialdehyde molecules with Cytosine; Malondialdehyde-x3-Cytosine
MALDI-TOF/TOF	Matrix-Assisted Laser Desorption/Ionization tandem time-of-flight
MeAαC	2-amino-3-methyl-9H-pyrido[2,3-b]indole
MeOH	Methanol
MDA	Malondialdehyde
MeG	Methylguanine
MelQ	2-amino-3,4-dimethylimidazo[4,5-f]quinolone
MeIQx	2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline
MGMT	O^6-methylguanine-DNA methyltransferase
Min	Minutes
MS	Mass Spectrometry
MS/MS	Tandem MS
N^7-MeG	N^7-methylguanine
N_2O_3	Nitrous anhydride
NCE	Normalized Collision Energy
Neu5Gc	N-glycolylneuraminic acid
NMR	Nuclear Magnetic Resonance
NO	Nitric Oxide
NOC(s)	N-Nitroso Compound(s)

NO_2Cl	Nitryl Chloride
$^1O_2$	Singlet oxygen
O_2^-	Superoxide
O^4-eT	O^4-ethylthymine
O^6-CMdG	O^6-carboxymethyl-2'-deoxyguanosine
O^6-CMG	O^6-carboxymethylguanine
O^6-MedG	O^6-methyl-2'-deoxyguanosine
O^6-MeG	O^6-methylguanine
Oct-G	Octenal-Guanine
OHE-C	Oxohexenal-Cytosine
OH-G	Hydroxyguanine
$ONOO^-$	Peroxynitrite
OPLS-DA	Orthogonal Partial Least Squares-Discriminant Analysis
PAHs	Polycyclic Aromatic Hydrocarbons
PBS	Phosphate Buffered Saline
PCA	Principal Component Analysis
Pen-G	Pentenal-Guanine
PhIP	2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine
PUFA(s)	Polyunsaturated fatty acid(s)
QTOF	Quadrupole – time-of-flight
QqQ-MS	Triple Quadrupole Mass Spectrometer
ROS	Reactive Oxygen Species
RT	Retention time
SCFA(s)	Short Chain Fatty Acid(s)
s.e.	Standard Error
SHIME	Simulator of the Human Intestinal Microbial Ecosystem
SIM	Selected Ion Monitoring
SPE	Solid Phase Extraction
spp.	Species
SRM	Selected Reaction Monitoring
T	Thymine
T0	Pre-colonic digestion sampling time point
T48	Post-colonic digestion sampling time point

TBARS	Thiobarbituric Acid Reactive Substances
TE or Tris-EDTA	Tris(hydroxymethyl)aminomethane - Ethylenediaminetetra-acetic acid (buffer)
TOF	Time-of-flight
TOF/TOF	Tandem TOF
TrpP2	3-amino-1-methyl-5H-pyrido[4,3-b]indole
Trypsin-EDTA	Trypsin - Ethylenediaminetetra-acetic acid (buffer)
U	Uracil
(U)HPLC	(Ultra)High Performance Liquid Chromatography
UV-VIS	Ultra Violet – Visible Spectroscopy
VIP	Variable Importance in Projection
WCRF	World Cancer Research Fund
WHO	World Health Organization

CHAPTER I

General introduction

In part adapted from:

Hemeryck LY, Moore SA, Vanhaecke L. Mass Spectrometric Mapping of the DNA Adductome as a Means to Study Genotoxin Exposure, Metabolism, and Effect. Anal Chem. 2016 Aug 2;88(15):7436-46. | p. 9-12 & 42-50.

&

Hemeryck LY, Vanhaecke L. Diet-related DNA adduct formation in relation to carcinogenesis. Nutr Rev. 2016 Aug;74(8):475-89. | p. 13-34.

1. DIET AND CANCER

1.1 Cancer facts and statistics

Cancer is the second cause of death worldwide [1], rendering it to be one of the most devastating diseases one can encounter in human life. The disease lays a very heavy burden on the afflicted individual as well as his or her loved ones. Furthermore, besides the unmistakable social impact, cancer also comprises a very high economic impact, not only for the patient, but also for society as a whole. In fact, compared to any other worldwide occurring cause of death, cancer accounts for the highest drain on economy due to direct medical costs and loss of productivity because of (temporary or permanent) disability and premature death [2].

In 2012, an estimated number of 1.4 million new cancer cases and 8.2 million cancer deaths occurred globally. Moreover, the International Agency for Research on Cancer (IARC) predicts an ever-increasing global cancer burden over the next few decades, soon reaching a total number of more than 20 million new cancer cases per year [1]. Improved cancer diagnosis and treatment has led to a vast decline in cancer mortality. In fact, overall cancer survival has doubled in the last 40 years [3]. In contrast, overall cancer incidence is on the rise, which is mainly caused by the increase in overall life expectancy; the longer people live, the bigger the chance of the occurrence and accumulation of mutations that lead to the possible onset for cancer initiation, promotion and progression (see figure 1 and section 1.2.3).

With a worldwide incidence of respectively 13 %, 11.9 % and 9.7 %, lung cancer, breast cancer and colorectal cancer (CRC) represent the most prevalent types of cancer (figure 2). Combined with prostate, liver and stomach cancer; those 3 main cancer types contribute to 55 % of global cancer incidence. In more developed regions, lung, breast, colorectal and prostate cancer account for half of the total cancer incidence, whereas in less developed regions, lung, breast, colorectal and prostate cancer, combined with liver and cervical cancer contribute to just over half of total cancer incidence. In Belgium, breast cancer is the most prevalent cancer type, followed by prostate cancer and CRC (figure 3) [1].

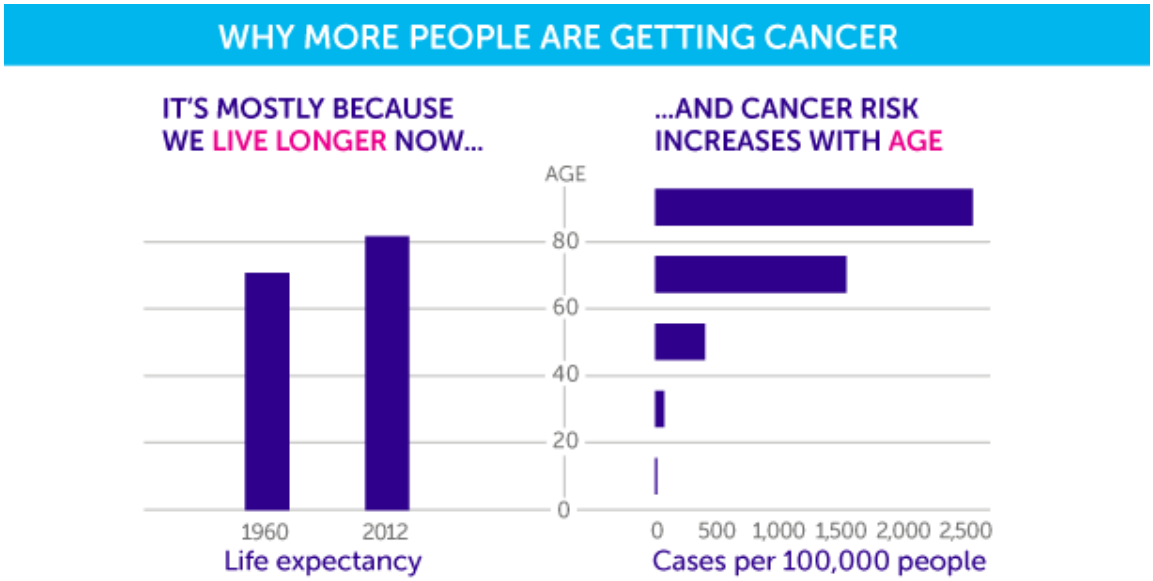

Figure 1. Since cancer risk increases with age, long life expectancy causes an increased cancer incidence [3].

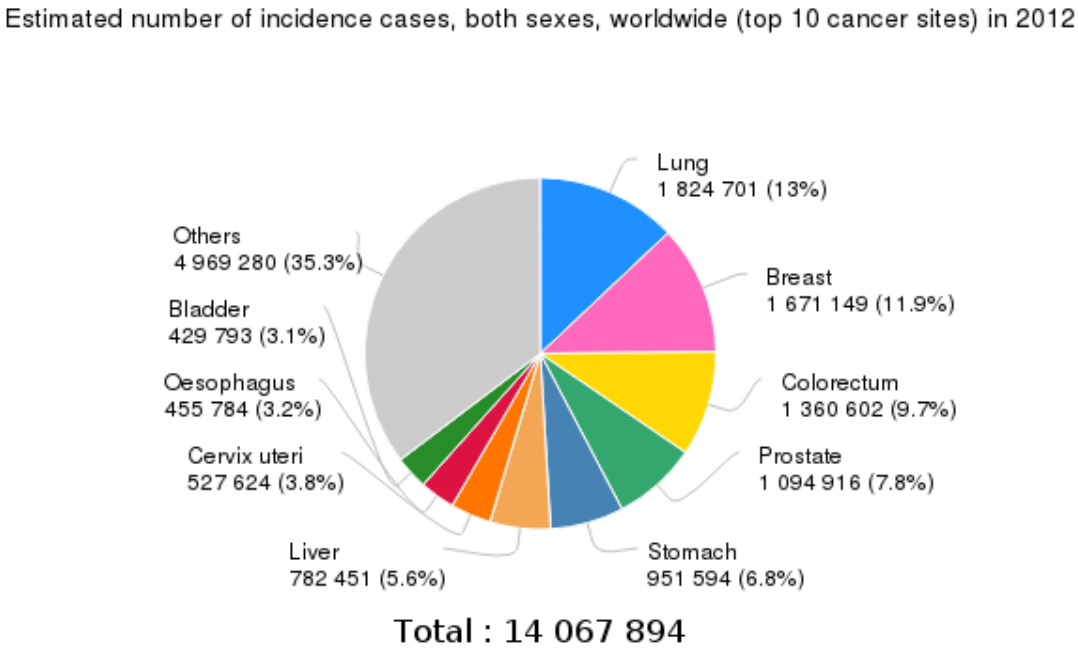

Figure 2. Worldwide cancer incidence in 2012 [1].

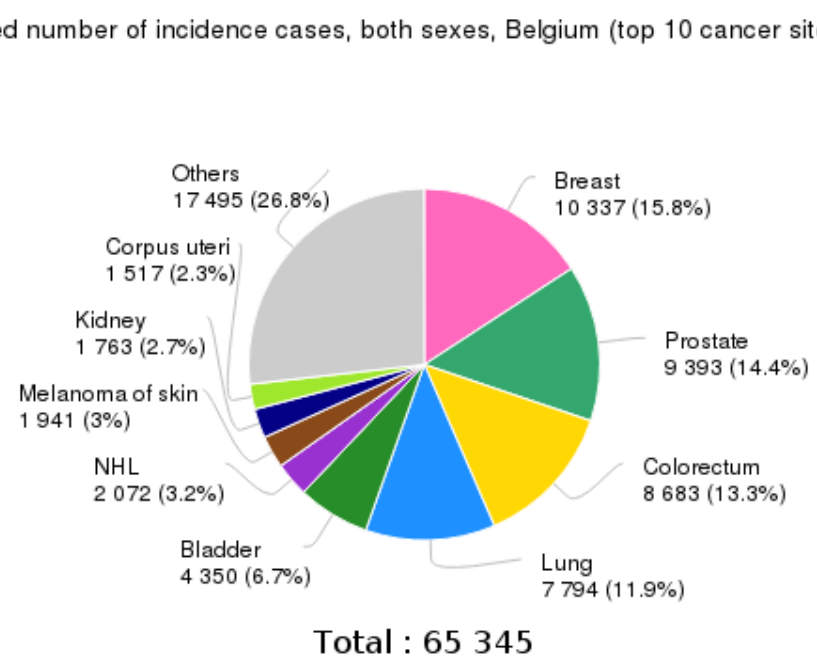

Figure 3. Belgian cancer incidence in 2012 [1].

1.2 The origin(s) of cancer

1.2.1 Cancer history in brief

The earliest written documentation of cancer dates back to an Egyptian scroll from 3000 BC, the Edwin Smith Papyrus, describing a case of breast cancer; 'cool to the touch, bulging and spreading all over the breast' [4]. Throughout history the 'what?' and 'how?' of cancer was a much-debated subject. Ancient Egyptians merely blamed the gods, whilst Hippocrates (400 BC) believed an excess of 'black bile' (one of 'the 4 body fluids') was the underlying cause of cancer [5]. As time went by and medicine evolved, scientists developed many different hypotheses (some more plausible than others), but it wasn't until the 18th and 19th century that the foundation for cancer epidemiology and modern oncology was laid. Invention of the modern microscope, implementation of autopsies (which required 'rebellion' against religious beliefs), and the possibility to perform surgery with anesthesia significantly contributed to the ever-evolving field of oncology [6-8].

1.2.2 The cause(s) of cancer

Percival Pott, an English physician from the 18th century, was the first to hypothesize and confirm that cancer can be caused by exposure to environmentally occurring carcinogens by linking the exposure to chimney soot (formed due to the incomplete combustion of e.g. coals) to the prevalence of scrotal cancer in chimney sweepers [9]. Nowadays, it has become clear that environmental carcinogens constitute the main cause of cancer because approximately 90 % of cancer deaths cannot be attributed to genetics [10]. Unfortunately, for the vast majority of non-hereditary cancers, the causative exposures and exact mechanism of cancer initiation, promotion and progression are highly complex, contributing to the fact that, even up to date, the underlying mechanisms remain mostly unexplained. Environmental exposures are difficult to track and characterize. Hence, with regard to the initiation and/or promotion of cancer, it is difficult to fully grasp the key exposures [11]. According to the World Health Organization (WHO), most cancers are associated with environmental, lifestyle, or behavioral exposures. This includes smoking of tobacco, consumption of alcohol, infections, reproductive and hormonal factors, diet, obesity, physical activity, occupation, (ionizing, ultraviolet and electromagnetic) radiation, (air, water and soil) pollution, pharmaceutical drugs, and naturally occurring chemical carcinogens through different routes of exposure [12].

1.2.3 The different stages in cancer development

The development of cancer constitutes a complex multi-step process that is characterized by the unregulated division of 'abnormal' cells. This multi-step process can be divided into four sequential steps: tumor initiation, tumor promotion, malignant conversion, and tumor progression.

During the initial phase of initiation, a permanent genetic change in proto-oncogenes and/or tumor suppressor genes (i.e. genes that 'manage' normal cell division) occurs. For chemically induced carcinogenesis, the formation of carcinogen-DNA adducts appears to be a necessary prerequisite for tumor initiation. DNA adduct formation that leads to the activation of a proto-oncogene or the inactivation of a tumor-suppressor gene can thus initiate carcinogenesis. The initiating carcinogen can be intrinsically genotoxic or requires prior activation during metabolisation in the body itself [13, 14].

After initiation, a selective clonal expansion of initiated cells under the influence of a 'promotor' can induce the second phase in cancer development; tumor promotion. During the phase of promotion, the clonal expansion of initiated cells will eventually lead to the production of a larger population of cells that are at risk of further genetic changes and malignant conversion. During this phase, frequent exposure to one or more promotors appears to be more important than exposure to a single high dose. Promotion will eventually give rise to the growth of benign or premalignant lesions [13].

Malignant conversion, the third phase in cancer development (not always considered as a separate phase), requires transformation of a pre-neoplastic cell to a neoplastic cell that expresses a malignant phenotype. This requires further genetic changes, of which the probability is in fact considerably low. Those genetic changes can be caused by DNA-damaging agents or errors during DNA replication (the latter is less common than the first) [13].

In the 'final' phase of tumor development, the tumor progresses in a more aggressive manner. Tumor cells express a malignant phenotype and begin to grow uncontrollably, possibly also invading surrounding tissues. Furthermore, this can be followed by dissemination of tumor cells from the primary tumor location, with the occurrence of regional and distant metastasis [13, 15].

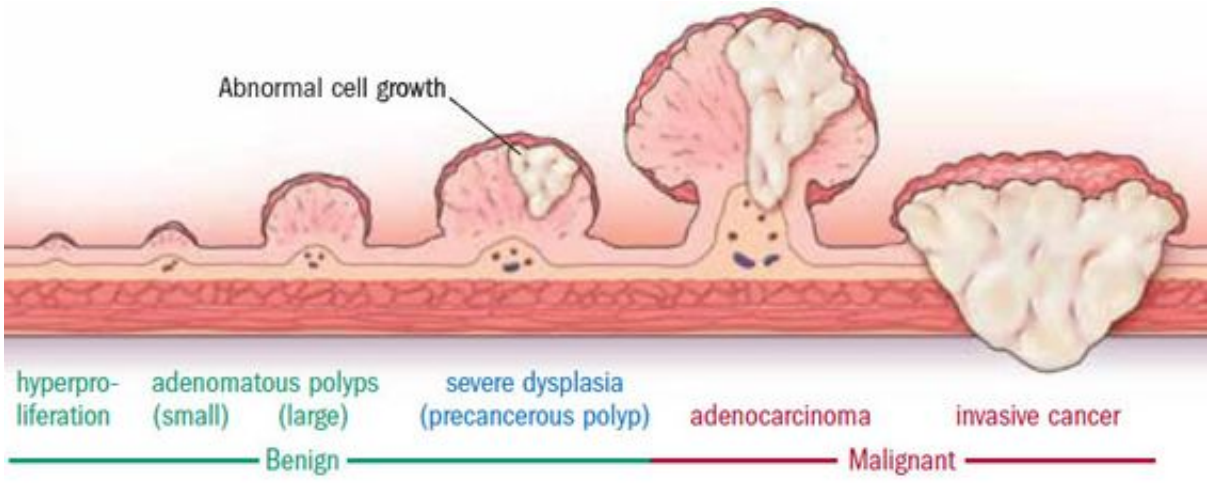

Figure 4. Colorectal cancer progression [16].

1.3 The human diet: the good, the bad and the ugly

Food is vital, but many of the regularly consumed foodstuffs are rather 'unhealthy', and possibly even pro-carcinogenic. Since food is an important part of our day-to-day lives, investigation of the relation between the human diet and initiation and/or progression of disease is extremely relevant. Therefore, causal links between diet and the possible onset of disease has always been of particular interest. After all, only if the underlying mechanisms can be unraveled, preventive strategies and guidelines can be developed to reduce the burden of an 'unhealthy' diet [12].

Over the years, a confident link between the consumption of a certain diet (or specific foodstuff) and the development of certain types of cancers has been established. For example, the 'Western diet' (high intake of fat, sugar and meat) has been implemented in the development of CRC, breast cancer and prostate cancer, whilst alcoholic beverage consumption has been linked to for example, cancer of the oral cavity, larynx, pharynx, oesophagus and liver [17].

The 'what?' and 'how?' behind the diet-related development of cancer can occur *via* different routes of exposure and molecular mechanisms. First of all, dietary genotoxins can occur naturally because of their direct or indirect natural occurrence (e.g. safrole is inherent to e.g. nutmeg, black pepper, and cinnamon [18] (direct exposure), ptaquiloside residues can occur in milk from bracken-fed cattle [19] (indirect exposure)). Secondly, the foodstuff at hand can contain carcinogenic chemicals due to contamination during one of the many stages of food production (e.g. mycotoxins produced by molds on grains [20], heterocyclic amines (HCAs) formed during high-temperature cooking of meat [21]). Thirdly, and lastly, the digestion and metabolisation of food can also (erroneously) lead to the (increased) endogenous formation of carcinogenic metabolites. For example, the gut microbiota play a significant role in the transformation of xenobiotics, which can lead to *de novo* production of toxins [22]. Exposure to environmental carcinogens can thus occur *via* different pathways, and continuously throughout one's life. Of course, whether or not dietary exposure to carcinogens indeed induces the initiation, promotion, malignant transformation, and progression of cancer depends on several other factors including the level and extent of exposure, individual rates of metabolisation/detoxification, the rate of mutagenicity of the genotoxin at hand, the exact location of a mayhap induced mutation (in proto-oncogenes or not), and efficiency of DNA repair [23].

2. THE DNA ADDUCTOME

2.1 The DNA adductome as a part of the exposome

Assessment of the exposure to the large spectrum of environmental factors and their direct or indirect role in the onset and further development of disease has proven to be a challenge. In 2005, Wild introduced the 'exposome' as a complementary concept to the 'genome'; a term that was introduced at least 10 years before [24]. The genome contains the whole of all genetic material of a certain organism (encompassing all of its inheritable traits), whilst the exposome encompasses all of the encountered exposures of a certain individual over the course of its, his or her lifetime, from the very early stages of conception and embryonic development through to adulthood, old age and death. In contrast to the individual genome, which is set at conception, the individual exposome evolves continuously throughout one's life [11, 24-26].

In light of genotoxicity and carcinogenicity studies, the DNA adductome, which consists of all DNA adduct types and levels present in a certain DNA sample, and can be considered as a part of the exposome, is of particular interest. DNA adducts originate from the interaction and subsequent covalent bonding between an electrophilic molecule and nucleophilic sites in DNA (i.e. the guanine (G), adenine (A), cytosine (C) and thymine (T) nucleobases) [27, 28]. The majority of DNA reactive molecules have the potential to lead to mutations and chromosomal alterations during DNA replication *via* formation of DNA adducts or DNA strand breaks, thus possibly resulting in carcinogenesis later on [29]. Therefore, DNA adduct formation is deemed to be the first step in chemically induced carcinogenesis [27].

2.2 DNA adduct analysis as a means to study genotoxin exposure, metabolism and effect

DNA adducts originate from the exposure of cellular DNA to endo- as well as exogenous genotoxins. Tissues and cells are exposed to endogenously generated chemicals through several (patho)physiological processes on a daily basis, including attack of DNA by e.g. reactive oxygen and carbonyl species, lipid peroxidation products (LPOs), estrogens and S-adenosylmethionine (gene expression regulator and methyl donor). [30]. However, in toxicology and cancer risk assessment the exogenous exposure to xenobiotics is deemed more important [31]. Examples of exogenous DNA adduct formation consists of DNA damage by dietary toxins such as

mycotoxins, acrylamide and HCAs [32-34]. In addition, several other environmental lifestyle factors can also significantly contribute to genotoxin exposure and exogenous DNA adduct formation; e.g. smoking, alcohol, certain industrial occupations, and living conditions [35-37].

The direct measurement of genotoxic chemicals in body tissues and fluids does not take into account important factors such as interindividual differences in exposure, absorption and distribution. Moreover, these chemicals may have a rapid turnover in the body, making direct measurement impossible. Hence, assessment of the DNA adductome offers a more thorough view of the different biological pathways involved in genotoxin exposure. This is especially pertinent since individual heterogeneity in genotoxin metabolism and DNA repair specifically complicates a straightforward assessment of the effect of certain genotoxins. Accordingly, holistic assessment of all DNA adduct types and levels ('DNA adductome mapping') provides a more appropriate tool to study the biological effect of a genotoxic chemical. The arguments raised above demonstrate the fact that DNA adducts show great potential as 'biomarkers of exposure' or 'biomarkers of internal dose'. Furthermore, since DNA adducts represent the amount of genotoxin that 'successfully' reached the DNA molecule in a certain individual, they can even act as a 'biomarker of the biological effective dose' of a certain genotoxic substance for that particular individual [38, 39].

Although interpretation of DNA adduct formation is complicated by several interfering factors, the field of DNA adductomics shows great potential in different areas of research. DNA adductome mapping does not only enable research into genotoxin exposure, but can also provide information on interindividual differences in genotoxin detoxification or activation. For example, Haugen and colleagues demonstrated a gender related difference in susceptibility to DNA adduct formation in tobacco smokers due to a significantly higher expression level of lung cytochrome P450 1A1 in women [40]. Genetic polymorphisms in DNA repair can also be a source of interindividual variation in DNA adduct levels [39]; e.g. Xia and co-workers recently published research on interindividual differences in aflatoxin B_1 (AFB_1) DNA adduct formation due to certain genetic polymorphisms in a DNA repair gene [41].

In addition to information on exposure to genotoxins, interindividual differences in genotoxin metabolisation and individual susceptibility to DNA damage and repair, DNA adduct analysis also provides important evidence on the possible long-term adverse health effects of genotoxic chemicals. When DNA adducts are introduced to the DNA sequence, the resulting DNA

damage may lead to mutagenesis and carcinogenesis [29]. At the time, there are at least three cases in which a causal link between the occurrence of DNA adducts and cancer incidence have been confirmed; firstly, aflatoxin B_1 DNA adducts and their link to hepatocellular carcinoma [42]; secondly, the case of polycyclic aromatic hydrocarbon DNA adducts and cervical cancer [43], and thirdly, the link between aristolochic acid consumption (*via* consumption of Aristolochia plants), aristolactam DNA adduct formation and transitional cell (urothelial) carcinoma of the upper urinary tract [44]. For many other exo- or endogenous DNA adducts, potential clues for the probable relationship between DNA adduct levels in tissue and cancer incidence are accumulating, emphasizing the significance of DNA adduct studies in the field of toxicology and cancer epidemiology [38, 39]. DNA adduct research may not provide the full answer but can certainly aid with the elucidation of cancer susceptibility and mechanisms, and potentially lead to improved cancer prevention and/or development of treatments for at-risk individuals. In figure 5, the position of DNA adduct formation in the pathway of genotoxin exposure, metabolism and effect is presented to illustrate the potential use of DNA adduct analysis for in-depth assessment of genotoxin exposure, metabolism and effect.

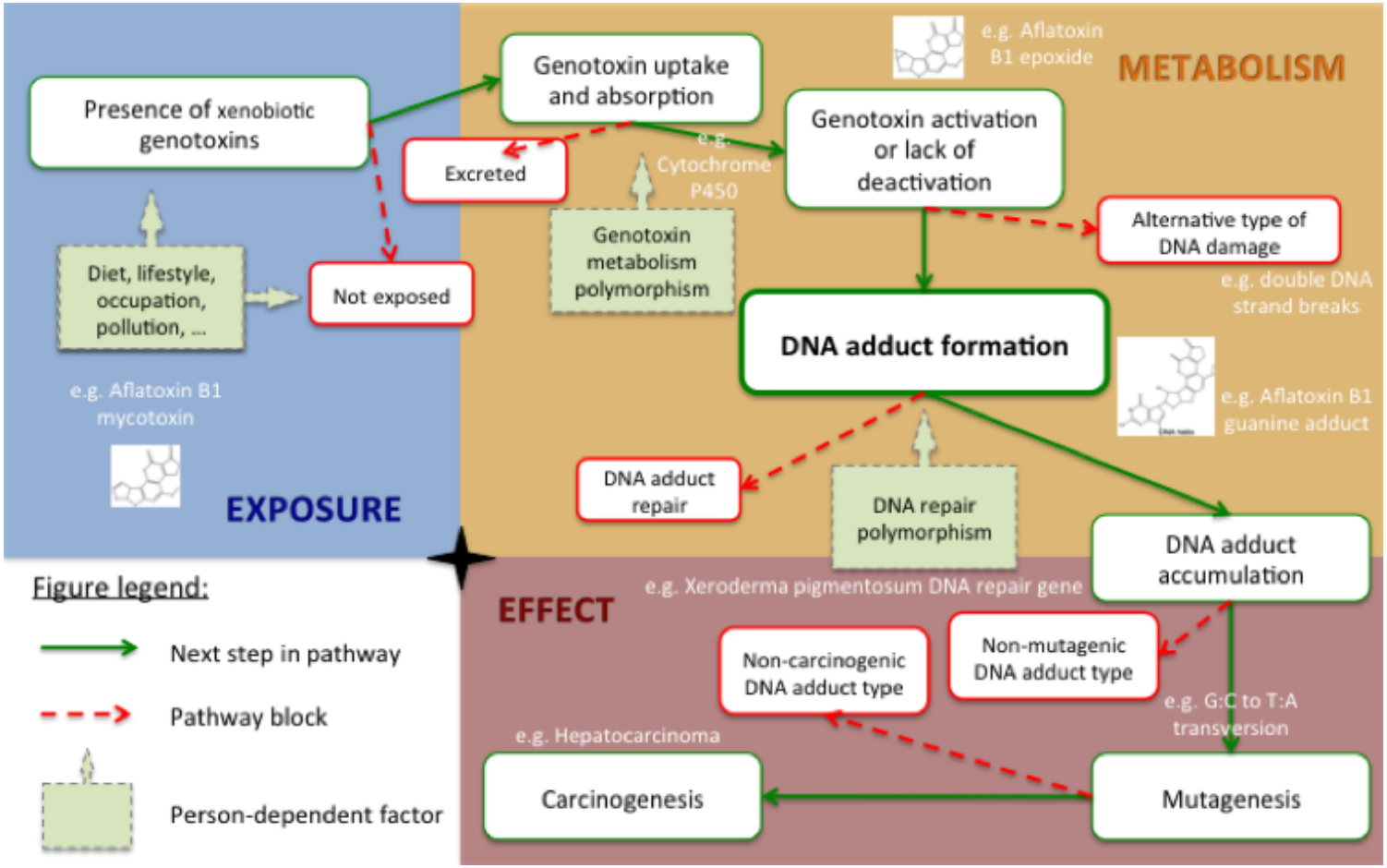

Figure 5. DNA adducts in relation to genotoxin exposure, metabolism and effect (aflatoxin B_1 associated hepatocarcinogenesis as a case study).

3. DIET-RELATED DNA ADDUCT FORMATION IN RELATION TO CARCINOGENESIS

Although the regular consumption of food is an absolute necessity, several foods and foodstuffs can also exert adverse health effects; the human diet, which is quite variable and complex, contains numerous beverages and food sources that consist of various ingredients and contaminants that may harbor mutagenic and carcinogenic toxicants. The uptake of those genotoxins *via* food can result in diet-related DNA adduct formation (figure 6), possibly even on a daily basis.

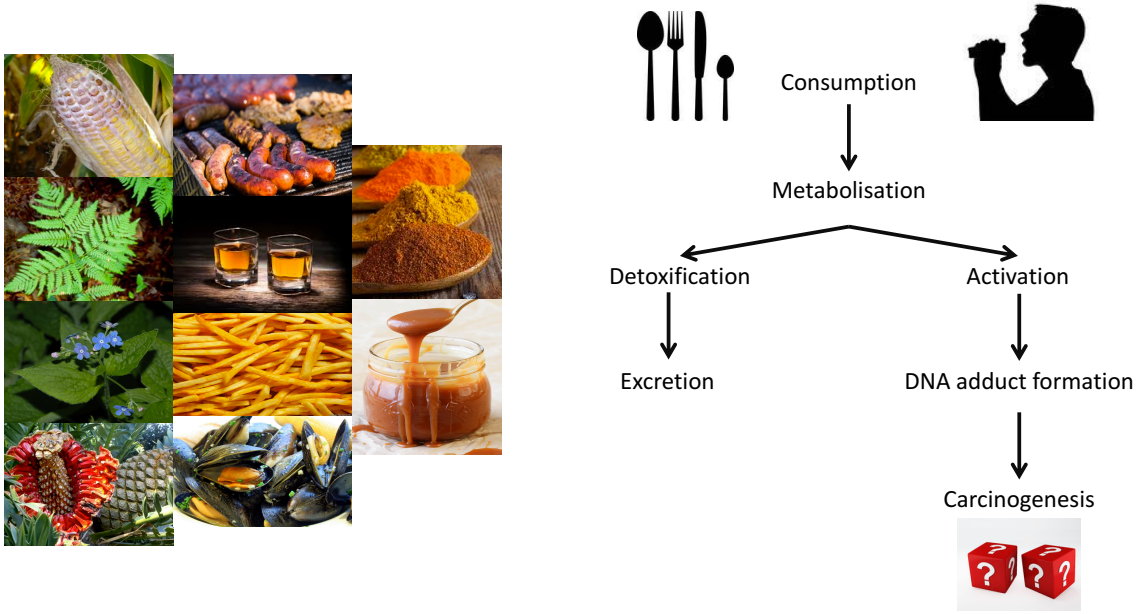

Figure 6. DNA adduct formation in the pathways that link diet and carcinogenesis.

An extensive literature review was executed to assess the current knowledge on diet-related DNA adduct formation. What follows, is a comprehensive overview and several examples of (groups of) diet-related DNA adducts that are or have been under investigation. In addition, the mutagenic and carcinogenic role of the described diet-related DNA adducts is explored to question or affirm the proposed causality between diet, DNA adduct formation and carcinogenesis.

3.1 Naturally Occurring Toxins

Several potent toxins are naturally present in food and/or feed, some of which are genotoxic and known to induce DNA adduct formation upon ingestion, as discussed below.

3.1.1 Mycotoxins in food and feed

The first group of diet-related DNA adducts are those generated by mycotoxins. Mycotoxins are secondary, toxic metabolites produced by food and feed-contaminating fungi. Several mycotoxins are well known for their diverse adverse health effects [20]. Aflatoxins, sterigmatocystin, ochratoxin, fumonisin, zearalenone, and some other toxins produced by *Penicillium spp.* are mycotoxins that demonstrate carcinogenic potential upon ingestion [32]. Nonetheless, only one mycotoxin, Aflatoxin B_1 (AFB_1), has been classified as a human carcinogen by the International Agency for Research on Cancer [45]. The AFB_1 mycotoxin appears to be a potent carcinogen in different species and different organs, such as the liver (major target organ), lungs, kidneys, and colon. Exposure to AFB_1 can lead to the formation of different AFB_1-specific DNA adducts but can also increase levels of nonspecific endogenous DNA adducts like 8-hydroxyguanine (8-OH-G) [32]. AFB_1-N^7-G is the major AFB_1 DNA adduct and is an established biomarker of AFB_1 exposure and effect, since research has definitively linked the prevalence of AFB_1-N^7-G in liver DNA to the incidence of hepatocellular carcinoma [31, 42, 46].

Aflatoxin M_1, an AFB_1 metabolite, is also able to bind to DNA, but to a lesser extent compared with AFB_1. DNA adduct formation by dietary exposure to sterigmatocystin, ochratoxin A, and zearalenone has been reported as well [47-49], although structural elucidation of the DNA adducts formed is still lacking. Nonetheless, some of these mycotoxins can generate aspecifc DNA adduct formation through the promotion of lipid peroxidation (see 3.4 Oxidative DNA damage below), which is the case not only for the previously discussed AFB_1 but also for other compounds, e.g. ochratoxin A and the T-2 trichothecene mycotoxin [32, 50, 51].

3.1.2 Pyrrolizidine alkaloids

Pyrrolizidine alkaloids are secondary plant metabolites known for induction of hepatotoxicity, carcinogenicity, genotoxicity, teratogenicity, and pneumotoxicity. Pyrrolizidine alkaloid–containing plants are widely spread across the globe (13 different plant families and 3 % of all flowering plants) and are often associated with poisoning of livestock, wildlife, and humans [52]. The presence of pyrrolizidine alkaloids has been reported in milk, honey, eggs, meat, salads, medicinal herbs, bread, grains, cereal, tea, and other beverages [29, 52, 53]. Adduction to proteins and DNA by pyrrolizidine alkaloid metabolites (e.g. dehydropyrrolizidine) is deemed responsible for the acute, subacute, and chronic effects of this extensive group of plant compounds [29]. The carcinogenic effects of pyrrolizidine metabolites have been demonstrated in different tissues and several animal models, and is associated at least in part with TP53 gene mutations [53]. Upon attack of DNA, the dehydropyrrolizidine molecules can induce at least 4 different DNA adducts with G as well as with A [54, 55].

3.1.3 Ptaquiloside in bracken fern

Ptaquiloside is the long-known major toxin in bracken fern (*Pteridium aquilinum*) [19, 56]. Both epidemiological and *in vivo* studies have illustrated the development of bladder and gastrointestinal tumors after chronic ptaquiloside exposure in animals and humans [56-58]. Although bracken ferns are not commonly included in the human diet, ferns were (or are still) consumed as the occasional wild food source in some regions or cultures [19, 59]. However, the most common way of human exposure appears to be aerial exposure to spores and the consumption of ptaquiloside-contaminated milk from bracken-fed cattle [19, 60].

Exposure to ptaquiloside has been linked to ptaquiloside-specific DNA adduct formation. Unstable and "active" breakdown products of ptaquiloside (ptaquilosin and ptaquilosin dienone) have DNA-alkylating properties and are most prone to attack A (at N^3) and G (at N^7) nucleobases, resulting in DNA adduct formation and DNA strand breaks [61]. Prakash et al. [62] demonstrated that grazing on bracken led to ptaquiloside exposure, ptaquiloside-DNA adduct formation, and H-ras mutations in the ileum of sheep. The carcinogenic effects of bracken and ptaquiloside through DNA adduct formation and H-ras proto-oncogene activation were further reinforced by follow-up research [63].

3.1.4 Azoxyglycosides in cycads

Cycads (*Cycadales*) are a species of (sub)tropical plants with historical economical, ornamental, and medicinal value [64]. Cycas seeds or nuts are a source of starch and are known to be consumed more frequently in times of famine (e.g. World War II). Some indigenous populations still use cycads during the preparation of certain traditional foods, although Westernization has significantly reduced this phenomenon [50, 65, 66]. Nevertheless, it is well known that Cycadales plant materials contain toxic azoxyglycosides such as cycasin and macrozamin, which can induce acute gastrointestinal complaints and chronic neurotoxic as well as genotoxic effects [67]. After ingestion, the azoxyglycosides are transformed to methylazoxymethanol aglycone (and its active methyldiazonium ion), eventually resulting in mutagenic, teratogenic, and carcinogenic effects *via* DNA alkylation in several species and organs [66, 68]. DNA adducts generated by methylazoxymethanol include O^6-methyl-G (O^6-MeG) and N^7-methyl-G (N^7-MeG) [66], although research on this topic appears to be quite sparse.

3.1.5 Herbs, spices, flavors and fragrances

Many foodstuffs contain herbs and spices to add flavor and fragrance. In some foods, the use of herbs and spices is omitted and replaced by the use of synthetic flavors and fragrances. Whether natural or synthetic, some of the chemical compounds that provide flavor and/or fragrance can induce genotoxic and carcinogenic effects.

Safrole is a naturally occurring plant compound present in sassafras oil, certain essential oils, and spices such as nutmeg, black pepper, and cinnamon [18]. Over the years, different epidemiological studies have linked safrole ingestion to DNA adduct formation and carcinogenesis (e.g. oral, esophageal, and liver cancer) [69-71]. The toxic effects of safrole appear to be associated with lipid peroxidation and oxidative DNA damage (please see 3.4 Oxidative DNA damage for more details on oxidative DNA damage), resulting in the formation of 8-OH-G, a nonspecific DNA adduct related to oxidative stress [72]. Safrole also induces, though less frequently, safrole-specific DNA adducts at the N^2 position of guanine and the N^6 position of adenine [69, 73]. Since safrole and related compounds such as dihydrosafrole and isosafrole have long been known to be hepatotoxic and carcinogenic [74], their use as additives is prohibited [75].

The flavoring substances estragole and methyleugenol are closely related to safrole (alkenylbenzenes). Estragole occurs naturally in tarragon, sweet basil, fennel, and anise. It can also be added to other foodstuffs like (non-)alcoholic beverages, canned fish, fats, and oils [76]. N^2-(trans-isoestragol-3′-yl)-G, N^2-(estragole-1′-yl)-G, 7-(trans-isoestragol-3′-yl)-G, and 8-(trans-isoestragol-3′-yl)-G are 4 different estragole-specific types of DNA adducts. Mainly N^2-(trans-isoestragol-3′-yl)-G has been linked to estragole's indirect genotoxicity and possible carcinogenicity [77]. Like estragole, methyleugenol occurs naturally in spices such as tarragon, fennel, basil, and anise, but it is also frequently added to baked foods, beverages, and candy. Methyleugenol consumption has been associated with the formation of DNA adducts [N^6-(trans-methylisoeugenol-3'-yl)-A and N^2-(trans-methylisoeugenol-3'-yl)-G], chronic hepatotoxicity, and hepatocarcinogenicity [78]. Additional examples of alkenylbenzene compounds that induce DNA adduction are myristicin and apiols, such as those from dill and parsley [79].

Reports on the contemporary medicinal use of plant extracts from Aristolochia spp. date back to the 1940s. Its ascribed anti-inflammatory activity can be attributed to the presence of aristolochic acids, which are a group of nitrophenanthrene carboxylic acids [80]. In 1982, however, Mengs et al. [81] revealed that aristolochic acid was strongly genotoxic and carcinogenic in rats. Follow-up research also revealed acute nephrotoxicity in rodents and carcinogenicity in mice [82, 83]. Although pharmaceutical preparations with aristolochic acid were withdrawn from the market in different countries about 3 decades ago and the use of plant extracts containing aristolochic acid was explicitly prohibited or strongly discouraged, the use of aristolochic acid extracts has not disappeared entirely [80]. An important clinical case example is that of several Belgian women who were admitted to the hospital with aristolochic acid nephropathy in 1993 and 1994 after ingesting aristolochic acid–containing Chinese herbs during a slimming therapy [84]. According to Schmeiser et al. and Arlt et al., aristolochic acid nephropathy and carcinogenicity is caused by DNA adduct formation [85, 86]. This is supported by the retrieval of specific aristolochic acid DNA adducts and AAG to TAG nonsense mutations (in codon 139 [Lys → Stop] of exon 5) in a urothelial tumor from a patient with aristolochic acid–associated nephropathy [87, 88]. A more recent and extensive study in Taiwan confirmed the significant contribution of aristolochic acid to aristolochic acid–related DNA adduct formation, p53 mutations, and the incidence of carcinomas of the upper urinary tract [89].

Other commonly naturally occuring or added flavors and fragrances are still under investigation. For example, Hossain et al. [90] recently investigated the DNA-damaging activities of several foodborne chemicals such as pyrogallol and gallic acid. The first is often found in smoked food because of the use of liquid smoke, and both compounds are commonly present in tea and coffee, the regular consumption of which is commonly associated with beneficial health effects. As such, pyrogallol is a perfect example to demonstrate the complex nature of dietary toxicity, since some individual foodborne chemicals seem to exhibit both DNA-damaging activity and health benefits [91, 92].

3.1.6 Marine toxins

Research on marine toxin DNA adducts is very limited and still ongoing. Exposure to marine toxins often occurs through consumption of fish and shellfish that either produce these toxins themselves or have accumulated them after initial production by algae. Okadaic acid is a marine toxin mainly associated with acute gastrointestinal complaints due to diarrhetic shellfish poisoning. Although a controversial topic, chronic exposure to lower concentrations of okadaic acid through consumption of shellfish appears to be associated with tumor induction caused by genotoxicity and DNA adduct formation [93]. The formation of DNA adducts 5-methyl-G and 8-OH-G is not specific for exposure of DNA to okadaic acid but does indicate okadaic acid genotoxicity as a result of alkylation and oxidation processes [94]. A different marine toxin, brevetoxin B (BTX-2), induces double DNA strand breaks, chromosomal alterations, and DNA adduct formation. However, exposure to this neurotoxin results mainly from inhalation of aerosols and not by ingestion [95]. Genotoxicity of additional marine toxins like domoic acid [96] has been reported, although no information on DNA adduct formation was available when this review was conducted.

Table 1. Confirmed DNA adducts originating from naturally occurring toxicants in food and feed.

Genotoxin group	Causal genotoxin	DNA adduct type
Mycotoxins [32]	Aflatoxin B_1	Aflatoxin B_1-G
		Aflatoxin B_1-A
		Aflatoxin B_1-C
		Aflatoxin-FAPY-G
		Hydroxy-G[a]
	Aflatoxin M_1	Aflatoxin M_1-G
	Aflatoxin P_1	Aflatoxin P_1-G
	Ochratoxin	Ochratoxin-G
	Sterigmatocystin	Sterigmatocystin-G
Pyrrolizidine alkaloids [54]	Pyrrolizidine alkaloid metabolites	Dehydropyrrolizidine-G
		Dehydropyrrolizidine-A
Azoxyglycosides in cycads [66]	Methylazoxymethanol aglycone	Methyl-G[a]
Herbs, spices, flavors & fragrances	Safrole [69, 72, 73]	Safrole-G
		Safrole-A
		Hydroxy-G[a]
	Isosafrole [97]	Isosafrole-G
	Estragole [76]	Estragole-G
		Estragole-A
	Methyleugenol [98]	Methyleugenol-G
		Methyleugenol-A
	Aristolochic acid metabolites [80]	Aristolactam I-G
		Aristolactam II-G
		Aristolactam I-A
		Aristolactam II-A
Marine toxins [94]	Okadaic acid	Methyl-G[a]
		Hydroxy-G[a]

[a]Nonspecific DNA adduct

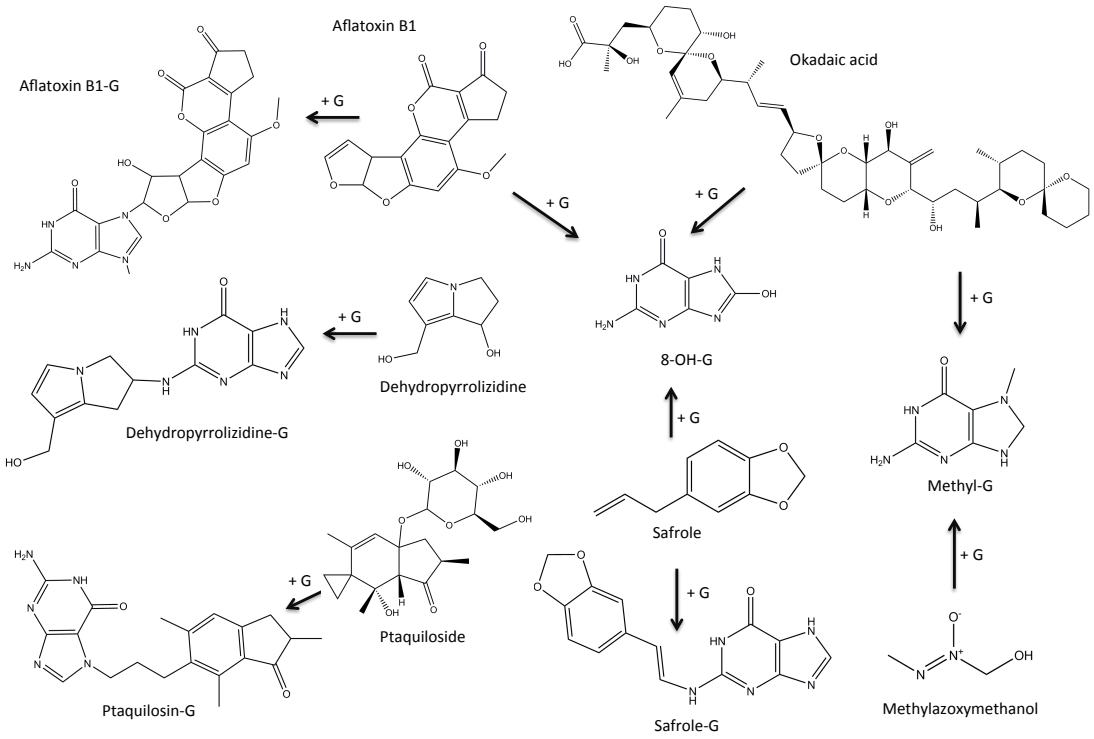

Figure 7. DNA adduct formation by some of the natural occurring toxins or their metabolites upon attack of the guanine (G) nucleobase.

3.2 Heating of foods

Different genotoxic compounds can be formed during extensive heating of food products. Several important and well-known examples are described below.

3.2.1 Acrylamide

Acrylamide is a genotoxic, tumor-promoting chemical frequently used in polyacrylamide polymer synthesis in laboratories and industrial settings [99]. It can be introduced into the human diet through contamination and, more importantly, through formation during high-temperature cooking (baking or frying) of starchy foods like potatoes and cereals. Acrylamide is a known neurotoxin and was classified as a probable human carcinogen by the International Agency for

Research on Cancer in 1994 [99, 100]. Although acrylamide seems to have a relatively low carcinogenic potential, its high concentrations (measured in parts per million) after baking or frying of starchy foods may pose a threat [100]. The carcinogenic potential of acrylamide and, in particular, its active metabolite glycidamide could be at least partially explained by the genotoxicity related to DNA adduct formation [101]. Possible acrylamide/glycidamide DNA adducts include carboxyethyl-G and carbamoylhydroxyethyl-G [33]. Nonetheless, the carcinogenic potential of acrylamide in humans remains controversial [102].

3.2.2 Furans

Furan and derivatives occur in a wide variety of heated food products like coffee, soy, caramel, bread, and meat [103]. These compounds give flavor to foods and are formed mainly by thermal degradation of pentoses. On the basis of hepatotoxicity and carcinogenicity in laboratory animals, the International Agency for Research on Cancer classified these compounds as possible human carcinogens [104]. Specific DNA adduct formation (e.g. N^2-((furan-2-yl)methyl)-G and N^6-((furan-2-yl)methyl)-A) by furans or furan metabolites has been described and may contribute to the furan carcinogenicity reported earlier [105, 106]. However, the presence of furans is not limited to heated foodstuffs. Furans are often used in industrial settings, which results in furan-polluted air and food. Because furans are dispersed in the environment, assessment of their effects from dietary intake exclusively is complicated.

3.2.3 Polycyclic aromatic hydrocarbons & heterocyclic amines

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental pollutants formed as a result of incomplete combustion of organic material. Exposure to PAHs is commonly linked to tobacco smoking and occupation-related air pollution (e.g. occupations at risk include workers in iron, steel, aluminum and coal production, traffic police, bus maintenance workers, chimney sweepers, etc.), although diet, i.e. consumption of charcoal-broiled meat, appears to be an important confounding factor in biomonitoring studies of occupational exposure to PAHs [107-109].

HCAs can be found in tobacco smoke condensates, diesel exhaust, and several food sources [21]. HCAs and PAHs are both well-known mutagens and carcinogens that occur in food in general

but are present at particularly high levels in meat cooked at high temperatures for a long period of time or held in or over an open flame [110].

Once HCAs are administered, their metabolization to N-hydroxy–heterocyclic amine derivatives is needed prior to formation of DNA adducts. Over 20 HCAs have been identified in foodstuffs, and several of them demonstrate DNA adduct formation at the C^8 or N^2 position of the G nucleobase. 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) is the most common HCA, while 2-amino-3,4-dimethylimidazo[4,5-f]quinolone (MelQ), 2-amino-3-methylimidazo[4,5-f]quinolone (IQ), and 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (8-MeIQx) are among the most potent bacterial mutagens ever tested (i.e., using the Ames test) [21]. Other HCAs with DNA adduction potential are 2-amino-3-methyl-imidazo[4,5-f]quinoxaline (IQx), 2-amino-3,4,8-trimethyl-3H-imidazo[4,5-f]quinoxaline (DiMeIQx), 2-amino-9H-pyrido[2-3-b]indole (AαC), 2-amino-3-methyl-9H-pyrido[2,3-b]indole (MeAαC), (2-amino-6-methyldipyrido[1,2-a:3,2-d]imidazole (GluP1), and 3-amino-1-methyl-5H-pyrido[4,3-b]indole (TrpP2). *In vitro* and *in vivo* mutagenicity, however, is dependent on species, tissue, cell, and type of HCA. Nevertheless, carcinogenicity of HCAs has been reported at low doses in several species [110], and although a causal link has not yet been established, the presence of HCAs in well-done meat products has been linked to, for example colon, stomach, and breast cancer [31]. The International Agency for Research on Cancer has classified 8 HCAs, among which MeIQ, MeIQx, and PhIP, as possible human carcinogens and IQ as a probable human carcinogen [45].

Interaction of PAHs with DNA is indirect; prior to DNA attack, PAHs must be activated (commonly by cytochrome P450 enzymes) to electrophilic epoxides. The most extensively studied carcinogen of the past century is the PAH benzo[a]pyrene [108]. An important finding is that benzo[a]pyrene DNA adducts in cervical tissues appear to be the missing link between exposure to PAHs through tobacco smoking and cervical cancer. Most studies focus on exposure to PAHs through occupation and tobacco smoking, but some studies demonstrate a link between charcoal-broiled meat consumption and PAH DNA adduct levels (trihydroxytetrahydro-benzo[a]pyrenelyl-G due to benzo[a]pyrene exposure (and conversion to the genotoxic benzo[a]pyrene-diolepoxide intermediate) in blood cells [109, 111]. The findings indicating that diet significantly contributes to PAH exposure, however, awaits scientific confirmation.

Table 2. Confirmed DNA adducts induced by known genotoxins in heated foods.

Genotoxin group	Causal genotoxin	DNA adduct type
Acrylamide [33]	Acrylamide	Carbamoylethyl-G
		Carbamoylhydroxyethyl-G
		Carboxyhydroxyethyl-A
		Carbamoylhydroxyethyl-A
		Carboxyethyl-G[a]
		Carboxyethyl-A[a]
		Carboxyethyl-C[a]
Furans	*cis*-2-butene-1,4-dial [105]	*cis*-butene-dial-G
		cis-butene-dial-A
		cis-butene-dial-C
	Furfuryl alcohol [106]	Methylfuran-G
		Methylfuran-A
	Hydroxymethylfurfural [112]	Furanformylmethyl-G
		Furanformylmethyl-A
Heterocyclic amines [34]	PhIP	PhIP-G
	PhIP	Hydroxy-PhIP-G
	IQ	IQ-G
	IQx	IQx-G
	MeIQ	MeIQ-G
	MeIQx	MeIQx-G
	DiMeIQx	DiMeIQx-G
	MeAαC	MeAαC-G
	AαC	AαC-G
	GluP1	GluP1-G
	TrpP2	TrpP2-G
Polycyclic aromatic [113]	B[a]P	B[a]P-G
		B[a]P-A
	B[a]PDE	B[a]PDE-G
		B[a]PDE-A

[a]Nonspecific DNA adduct

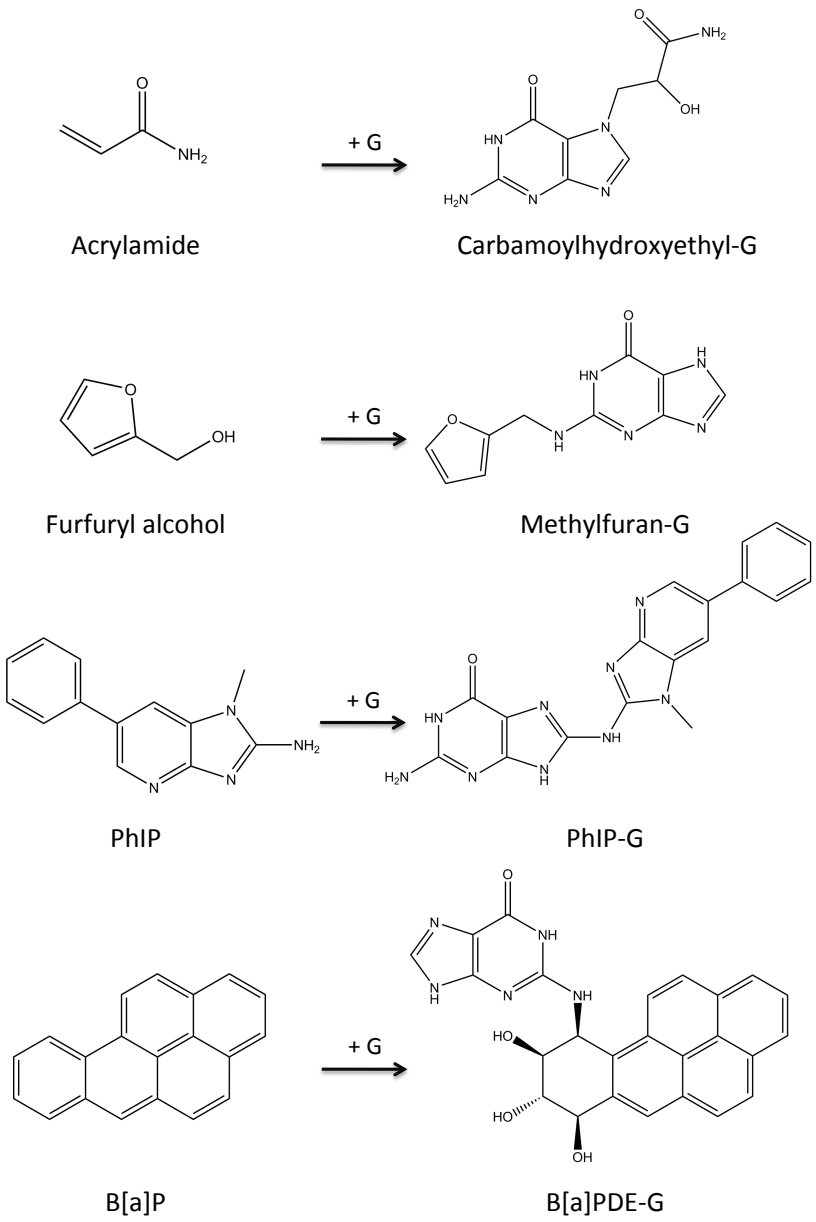

Figure 8. DNA adduct formation by 4 major heat-induced toxins in food upon attack of the guanine (G) nucleobase.

3.3 Alcohol

Ethanol consumption is a proven cause of cancer and has long been linked to the occurrence of hepatocellular carcinomas and squamous-cell carcinomas of the oral cavity, pharynx, larynx, and esophagus [114]. More recently, breast and CRC were added to that list [115]. As is the case for many cancers, the exact underlying etiology of alcohol-induced carcinogenesis is still unknown.

Animal studies demonstrated that acetaldehyde, the main metabolite of ethanol, can induce tumor formation in rats and hamsters, which led the IARC to classify acetaldehyde in group 2B, i.e. "possibly carcinogenic to humans," in 1999 [116]. A study conducted by Fang and Vaca [117] documented that acetaldehyde DNA adducts may be the missing link between alcohol consumption and different cancer types, since levels of acetaldehyde-generated DNA adducts in alcohol abusers were significantly higher than those in controls. Follow-up *in vitro* research showed that acetaldehyde induced DNA adduct formation in human buccal cells [118], and several other studies confirmed the formation of different DNA adducts by acetaldehyde *in vitro* [119, 120]. DNA adducts generated by acetaldehyde include N^2-ethyl-G, N^2-4-hydroxybutyl-G or N^2-3-hydroxybutyl-G and 1,N^2-propano-G [37]. Although acetaldehyde may contribute to ethanol toxicity and carcinogenesis, ethanol consumption also increases the production of reactive oxygen species and LPOs [121]. Both reactive oxygen species and LPOs are likely to attack DNA, providing the basis for an additional hypothesis on alcohol-related carcinogenesis. More details on DNA adduct formation by reactive oxygen species and LPOs are presented below. DNA adducts originating from direct and/or indirect alcohol genotoxicity are shown in table 3.

Table 3. DNA adducts formed by alcohol, reactive oxygen species, lipid peroxidation products, and alkylating compounds (e.g. N-nitroso compounds).

Genotoxin group	Causal genotoxin	Guanine damage	Adenine damage	Cytosine damage	Thymine damage
Alcohol [37]	Direct genotoxicity: acetaldehyde	Ethyl-G Ethylidene-G Etheno-G Hydroxybutyl-G Propano-G	Etheno-A	Etheno-C	
	Indirect genotoxicity: ROS	(see 'ROS (direct genotoxicity') & 'ROS (indirect genotoxicity)')			
ROS (direct genotoxicity) [30, 122, 127, 154]		Hydroxy-G Hydrohydroxy-G Fapy-G Oxazolone Carbamoyl-oxo-dihydroxyimidazolidine	Hydroxy-A Fapy-A	Hydroxy-C Hydroxy-U Dihydroxy-C Hydroxyhydro-C C-glycol U-glycol Alloxan Hydroxyhydantion	Hydroxyhydro-T Dihydroxy-U Hydroxyhydro-U Dihydro-U Dihydro-T T-glycol Formyl-U Hydroxymethylhydantion
ROS (indirect genotoxicity): LPOs [30, 37, 126, 155-157]	Malondialdehyde	M_1G (pyrimidopurinone-G) M_2G	M_1A (oxopropenyl-A) M_3A	M_1C (oxopropenyl-C) M_3C	
	Acetaldehyde	(see above; 'Alcohol')			
	Crotonaldehyde	Propano-G Etheno-G	Etheno-A	Etheno-C	

Table 3 continued (1).

Genotoxin group	Causal genotoxin	Guanine damage	Adenine damage	Cytosine damage	Thymine damage
ROS (indirect genotoxicity): LPOs *continued*	Pentenal	Pen-G			
	Hexenal	Hex-G			
	Heptenal	Hep-G			
	Octenal	Oct-G			
	Hydroxynonenal	HNE-G	Etheno-A	Etheno-C	
		Etheno-G			
	Malondialdehyde-acetaldehyde conjugates	M_1AA-G			
		M_2AA-G			
	Hydroxybutanal	Hydroxybutylidene-G			
		Paraldol-G			
	Methylglyoxal	Methylglyoxal-G			
	Glyoxal	Glyoxal-G			
		Glyoxal-C			
Alkylation [30, 142, 158-170]		Butyl-G	Butyl-A	Butyl-C	Butyl-T
		Carboxyethyl-G	Carboxyethyl-A	Carboxyethyl-C	Carboxyethyl-T
		Carboxy-G	Carboxyl-A	Carboxyl-C	Carboxy-T
		Carboxymethyl-G	Carboxymethyl-A	Carboxymethyl-C	Carboxymethyl-T
		Dimethyl-G	Dimethyl-A	Dimethyl-C	Dimethyl-T
		Ethyl-G	Ethyl-A	Ethyl-C	Ethyl-T
		Hydroxyethyl-G	Hydroxyethyl-A	Hydroxyethyl-C	Hydroxyethyl-T
		Hydroxymethyl-G	Hydroxymethyl-A	Hydroxymethyl-C	Hydroxymethyl-T
		Methoxymethyl-G	Methoxymethyl-A	Methoxymethyl-C	Methoxymethyl-T

Table 3 continued (2).

Genotoxin group	Causal genotoxin	Guanine damage	Adenine damage	Cytosine damage	Thymine damage
Alkylation *continued*		Methyl-G	Methyl-A	Methyl-C	Methyl-T
		Nitro-G	Nitro-A	Nitro-C	Nitro-T
		Propyl-G	Propyl-A	Propyl-C	Propyl-T
		Tetramethyl-G	Tetramethyl-A	Tetramethyl-C	Tetramethyl-T
		Trimethyl-G	Trimethyl-A	Trimethyl-C	Trimethyl-T

3.4 Oxidative DNA damage

Reactive oxygen species and reactive nitrogen species are formed continuously during the biochemical reactions of normal cellular metabolism. Reactive oxygen species (ROS) and reactive nitrogen species such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals ($HO^\cdot$), singlet oxygen ($^1O_2$), nitrous anhydride (N_2O_3), peroxynitrite ($ONOO^-$), nitryl chloride (NO_2Cl), and nitric oxides (NO) can directly or indirectly induce endogenous/background levels of altered nucleobases [122-124]. Examples of DNA adducts formed by the interaction of reactive oxygen species or reactive nitrogen species with DNA include 8-nitro-G, 8-OH-G, 2-hydroxy-A, thymine glycol, and cytosine glycol [30, 124].

ROS attack not only DNA but also other macromolecules such as lipids and proteins. When ROS and iron interact with lipids like polyunsaturated fatty acids in membrane phospholipids, LPOs are formed [121, 125]. In turn, those LPOs can exert both cytotoxic and genotoxic effects. During lipid peroxidation, different electrophilic epoxides and aldehydes are formed. A major LPO with mutagenic and carcinogenic properties is malondialdehyde (MDA). Attack of DNA by MDA primarily yields the DNA adduct M_1G, although M_1A, M_1C, and other DNA adduct types are formed as well [126, 127]. Additional examples of lipid peroxidation end products with DNA-damaging properties are acetaldehyde, malondialdehyde-acetaldehyde conjugates, hydroxynonenal, crotonaldehyde, and acrolein [37, 125].

Both LPOs and their resulting DNA adducts can occur endogenously following production of reactive oxygen species or reactive nitrogen species during physiological oxidative stress [122, 128]. On the other hand, several exogenous or pathophysiological sources of oxidative stress, such as immune responses, chronic inflammation, radiation, chemicals, drugs, and dietary imbalance, can also contribute to oxidation of DNA [125, 128, 129]. Dietary imbalance can result from the overconsumption of energy, dietary fat (polyunsaturated fatty acids, in particular), and red and processed meat, which has been associated with an elevated incidence of chronic diseases like chronic pancreatitis, coronary heart disease, inflammatory bowel disease, diabetes, and cancer (e.g. oral, pharyngeal, stomach, prostate, pancreatic, and colon) [129-136]. Several studies have either (a) linked the occurrence of higher levels of oxidative stress, production of LPOs, and DNA adduct formation to the consumption or digestion of fat and meat, or (b) have established an association between the detection of increased levels of DNA adducts in different tissues and a documented disease status [125, 128, 129, 132, 137-141]. Follow-up studies could

provide more detailed information on diet–cancer causality and the exact underlying etiological mechanisms. An overview of oxidative stress–related DNA adduct formation is provided in table 3.

3.5 Alkylating N-nitroso compounds

Alkylating agents are genotoxic, mutagenic, and cytotoxic *via* interaction with DNA, RNA, and proteins. Some are ubiquitous or are formed endogenously during metabolism, while others are administered in high doses as chemotherapeutic agents [142]. DNA adducts generated by alkylation are unspecific, so at times, one can only speculate on the causal chemical [143]. Alkylation of DNA bases can occur at the O or N atom in DNA nucleobases and, in general, alkylation at O atoms of nucleobases is mutagenic and genotoxic, while N-alkylation leads predominantly to cytotoxic effects [142]. This is why considerable research has focused on the possible carcinogenic effects of adducts like O^6-MeG and O^6-carboxymethyl-G (O^6-CMG), although N^7-MeG and N^7-carboxymethyl-G are formed in higher concentrations upon attack of DNA by methylating and carboxymethylating agents, respectively [30, 144, 145].

Different types of chemicals have alkylating properties and some of them are diet or lifestyle related. Acrylamide, alcohol, and okadaic acid are examples that have already been described above, but an additional group of chemicals with DNA-alkylating properties are the endogenous or exogenous N-nitroso compounds (NOCs). The methylazoxymethanol compound, discussed previously, is an example of a naturally occurring exogenous NOC.

The presence of NOCs in foodstuffs and the accidental toxic effects were first noticed around 1964, when feeding of sodium nitrite–preserved herring caused hepatotoxicity in sheep [146]. Researchers later discovered that nitrosation of amines could lead to the formation of nitrosodimethylamine [147]. Confirmation of the carcinogenicity of nitrosodimethylamine followed shortly [148], and since then, the toxicity of many different NOCs in different species has been investigated thoroughly. Human exposure to NOCs can occur through occupation and lifestyle habits like smoking, but according to Lijinksy [147, 149], who has reviewed the occurrence, chemistry, and biology of NOCs extensively, diet represents the most important route of NOC exposure. Common foodstuffs containing NOCs are beer, certain spices, smoked fish and smoked fish products, and meat products such as smoked or cured pork, sausages and

fried bacon [150, 151]. In addition, besides NOCs present in foodstuffs, nitrosamines and nitrosamides can also be easily formed in the stomach by the interaction of nitric oxide or nitrite from metabolism, food, or saliva and secondary or tertiary amines from food or pharmaceutical drugs [123, 147]. A similar process can occur in the large bowel, where microbial fermentation of proteins leads to the production of amines. Nitrosation of those amines can then generate the formation of NOCs [152].

Tumor induction by nitrosamines or nitrosamides has been demonstrated in different tissues like liver, lung, kidney, bladder, tongue, bone, nervous system, and gastrointestinal tract [147]. Lung cancer is associated mainly with tobacco smoke and tobacco-specific NOCs, although dietary NOCs may contribute as well [153]. The exact mechanism of tumor induction and/or promotion by NOCs is not entirely clear; therefore, further research is warranted. Table 3 provides an overview of alkylation-induced DNA adducts.

3.6 Ingestion and cellular incorporation of DNA adducts from dietary origin

Food contains abundant genomic DNA of vegetable, fungal, and animal origin, including damaged DNA and, thus, DNA adducts. There is a broad range of damaged nucleotides/nucleosides/nucleobases in food, although the type of DNA lesions present depends on food species, growth conditions, and environmental parameters during food production processes. Following ingestion, these foodborne DNA lesions can be erroneously incorporated into human cellular DNA as a result of imperfections in DNA synthesis [171].

Research on possible adverse health effects of DNA damage in food is scarce, but the mutagenicity of certain externally administered DNA adducts has been reported [172-174]. Furthermore, it is worth noting that the administration of certain chemotherapeutic agents such as purine and pyrimidine analogs – which interfere with DNA synthesis in rapidly dividing malignant cells – is anything but new [175, 176]. Spilsberg et al. [171] recommended that novel and traditional foods should be screened for DNA adducts to gain more knowledge on this subject.

3.7 Discussion and conclusion

The modern human food chain includes countless foodstuffs and ingredients. Classification and determination of the biochemical activities and possible toxic effects of all bioactive molecules in the food chain seems unfeasible and highly complex. In addition, food can be easily contaminated with other environmental chemicals of non-food origin during the various stages of production, packaging, storage, and transport, thus rendering research of food-related toxicity and disease even less straightforward. Nevertheless, years of research on dietary toxicity have revealed the possible acute and chronic toxic effects of certain foodstuffs and their inherent bioactive molecules. As a result, some edibles have been excluded from the food web as much as possible (e.g. Aristolochia plant spp. extracts, because of aristolochic acid toxicity; discussed above), while others have been thoroughly evaluated and are considered safe (e.g. mushrooms containing hydrazine [116]).

In vitro and *in vivo* experiments have documented the formation of different types of DNA adducts as a result of exposure to certain dietary chemicals. The levels of those DNA adducts have been linked to certain pathophysiological processes like oxidative stress and chronic diseases including cancer [30, 125, 177]. Consequently, causal links between chemicals in food, DNA adduct formation, and carcinogenesis seem very likely, emphasizing the importance of further investigation of the exact origin and consequences of specific and nonspecific endogenous and exogenous DNA adduct formation.

Although DNA adduct studies can be complex, research on DNA adducts can provide useful insight into different physiological and pathophysiological pathways [38, 178]. Qualitative or quantitative assessment of xenobiotic chemicals in bodily matrices can reflect environmental exposure and the degree of intake and/or excretion, depending on the chemical and matrix at hand. DNA adduct levels, on the other hand, provide information on not only the exposure to and uptake of certain genotoxic chemicals but also the metabolization, detoxification, activation, distribution, and any resulting genotoxic effects of these chemicals [39]. As such, DNA adducts provide important clues on interindividual differences in genetic polymorphisms in DNA repair and susceptibility to cancer initiation and development [41, 177]. Hence, DNA adducts are not merely biomarkers of exposure but are also, more importantly, biomarkers of an internal dose or a biologically effective dose [38].

A DNA adduct of interest should be established as a biomarker of both exposure and effect to confidently link dietary genotoxin exposure to carcinogenesis through DNA adduct formation. To ratify a certain DNA adduct as a biomarker of effect, specific criteria must be met. For example, the prevalence of DNA adducts should be strongly, consistently, and specifically linked to disease outcome [107]. To date, the most prominent and confirmed example of pathogenic diet-related DNA adduct formation is the formation of AFB_1-N^7-G DNA adducts in the liver due to AFB_1 exposure and the subsequent onset of hepatocellular carcinoma [31]. Diet- and exposure-related DNA adduct formation is still under investigation for potential links between e.g. red meat consumption and the development of CRC [141, 177, 179], alcohol consumption and the development of cancers like hepatocarcinoma and colon carcinoma [180, 181], methyleugenol ingestion and hepatocarcinoma [182], and aristolochic acid exposure and urothelial cancer [183].

In conclusion, it can be stated that, although the puzzle is far from complete, evidence of the carcinogenic effects of diet-related DNA adduct formation is accumulating due to the fact that different sources of dietary genotoxins have been linked to both *in vivo* and *in vitro* DNA adduct formation. The in-depth investigation of diet-related genotoxin exposure, the therewith associated DNA adduct formation and disease outcome will provide further insight into diet- and lifestyle-related carcinogenesis.

4. INVESTIGATION OF THE COLORECTAL CANCER INITIATING AND/OR PROMOTING ROLE OF RED MEAT CONSUMPTION BY MEANS OF DNA ADDUCT ANALYSIS

4.1 Colorectal cancer

As was already mentioned before (1.1 Cancer facts and statistics), CRC is the third most common cancer type worldwide. More specifically, CRC is the third most common cancer in men, and the second most common in women. Almost 55 % of CRC cases occur in more developed regions (figure 9), reflecting the fact that CRC mainly is a 'Western' disease [1, 184]. Only 10 % of CRC cases are hereditary, which means the vast majority of CRC cases have a predominant environmental cause [12]. Composition of the diet, obesity and physical inactivity significantly contribute to CRC risk [12]. More importantly, about 70 % of CRC cases could be prevented by dietary and lifestyle changes [185, 186]. According to the World Cancer Research Fund (WCRF) and WHO, the consumption of red meat and processed meat, consumption of alcoholic beverages, body fatness, abdominal fatness, and factors leading to greater adult attained height (or its consequences) can cause CRC. In contrast, physical activity protects against colon cancer. Evidence also suggests that the consumption of garlic, milk, and calcium might also protect against CRC. Unfortunately, despite the convincing epidemiological evidence, the underlying, causative pathways have not yet been clarified and defined in detail [12, 187].

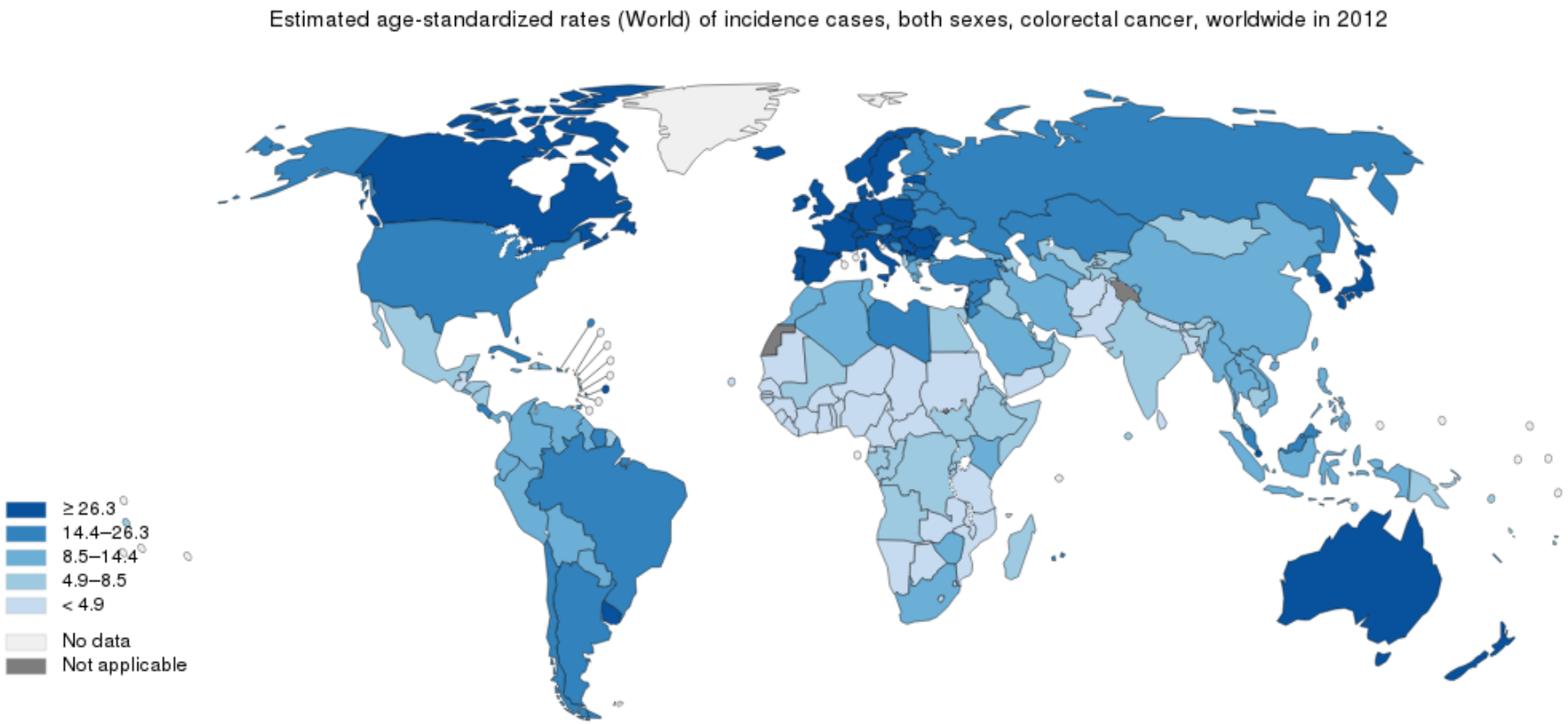

Figure 9. Estimation of worldwide colorectal cancer incidence for both sexes in 2012 [1].

Table 4 lists the relative risk of the major contributing dietary and lifestyle factors for CRC. Accordingly, several cancer research organizations and institutes (e.g. World Cancer Research Fund International) recommend to avoid smoking, avoid, and at least limit alcohol consumption, eat less than 500 g of red meat per week and avoid/limit processed meat consumption, but be 'physically active', maintain a healthy weight, and eat more reduced-fat dairy products, grains, vegetables, fruits and beans on their websites (www.wcrf.org and www.cancer.org).

Table 4. Relative colorectal cancer risk of the major contributing dietary and lifestyle factors [188].

Factor		Relative risk
Factors that increase risk		
	Alcohol consumption (heavy *vs.* nondrinkers)	1.6
	Obesity	1.2
	Red meat consumption	1.2
	Processed meat consumption	1.2
	Smoking (current *vs.* never)	1.2
Factors that decrease risk		
	Physical activity	0.7
	Dairy consumption	0.8
	Fruit consumption	0.9
	Vegetable consumption	0.9
	Total dietary fiber (10 g/day)	0.9

4.2 Evidence on the link between red and processed meat consumption and colorectal cancer

Epidemiological research has demonstrated a strong correlation between the consumption of red meat, and the consumption of processed meat, with CRC incidence and mortality. However, correlation does not necessarily reflect causation. Therefore, the hypothesized carcinogenic effect of meat consumption has always been, and still remains, a controversial topic. Nevertheless, on the 26th of October 2015, the IARC issued a press release stating that red meat is 'probably carcinogenic to humans' (group 2B) "based on limited evidence that the consumption of red meat causes cancer in humans and strong mechanistic evidence supporting a

carcinogenic effect", and that processed meat is 'carcinogenic to humans' (Group 1) "based on sufficient evidence in humans that the consumption of processed meat causes CRC". Red meat was defined as all types of mammalian muscle meat, such as beef, veal, pork, lamb, mutton, horse, and goat. Processed meat was defined as meat that has been transformed through salting, curing, fermentation, smoking, or other processes to enhance flavour or improve preservation. Most processed meats contain pork or beef, but processed meats may also contain other red meats, poultry, offal, or meat by-products such as blood [189].

4.3 Hypotheses on the link between red and processed meat consumption and colorectal cancer

Over the years, different hypotheses on red and processed meat carcinogenicity have been put forward. These hypotheses have been reviewed and discussed extensively over the years. To create an overview, the major hypotheses are discussed briefly below.

4.3.1 Refuted hypotheses

4.3.1.1 Total/saturated fat

A first hypothesis suggests that the high saturated fat content of red and processed meat, and/or the 'Western' diet in general, increases CRC risk through (1) an increased excretion of secondary bile acids, (2) an increase in free fatty acids, and/or (3) induction of a higher risk for obesity. Yet, several independent studies have not been able to confirm the link between the intake of total (saturated) fat and CRC promotion [190-193]. Anyway, other foodstuffs besides red and processed meat also contain high amounts of saturated animal fat (e.g. dairy products), which directly interferes with the hypothesis focusing on red and processed meat intake [191].

4.3.1.2 Protein

High total meat intake equals high protein intake. Excess protein is fermented in the large bowel, which results in the formation of toxic breakdown products (e.g. amines, phenols and H_2S). Hence, high total meat intake and excess protein intake was hypothesized to initiate and/or

promote CRC. However, in line with the hypothesis on total (saturated) fat, researchers have not been able to link total meat intake and/or excess protein fermentation in the gut to CRC risk. Rendering this hypothesis to fall short [130, 191, 193, 194].

4.3.2 Current hypotheses

4.3.2.1 The heme hypothesis

At the time, with regard to red meat, researchers are mainly focusing on one specific hypothesis; the heme hypothesis. The heme hypothesis is based on the fact that red meat (e.g. beef) contains a considerably higher amount of heme iron in comparison to white meat (e.g. poultry); red meat contains an at least 10-fold higher heme iron content under the form of the myoglobin protein, to which it owes its dark red color [190, 195, 196].

Pierre et al. were the first to document that the digestion of meat can promote carcinogenesis in the rat colon, dependent on heme concentration [197]. The exact underlying mechanism has not been elucidated yet, but it was hypothesized that heme iron catalyzes the formation of genotoxic NOCs, and cyto- and genotoxic LPOs [190]. In addition, heme may also exert direct toxic effects, although this is mostly uncharted at the time [191].

4.3.2.1.1 Catalyzation of oxidation

On multiple occasions, dietary heme has been linked to the urinary excretion of 1,4-dihydroxynonane mercapturic acid (DHN-MA), a lipid peroxidation marker [197, 198]. Hence, it was hypothesized that ingested heme iron catalyzes (lipid per)oxidation processes. Ferrous iron (cfr. heme iron) can stimulate the formation of ROS and LPOs through the self-maintaining Fenton reaction [199]. More specifically, Fe^{2+} and Fe^{3+} can respectively be oxidized and reduced to their respective counterparts Fe^{3+} and Fe^{2+} through interaction with H_2O_2, resulting in the production of hydroxyl and hydroperoxyl radicals. These ROS maintain a cascade of oxidative reactions, leading to the direct or indirect oxidation of macromolecules including lipids, proteins and DNA. As such, meat oxidation processes can interfere with CRC initiation and promotion *via* the induction of oxidative stress and/or DNA adduct formation [190, 191, 196, 200].

4.3.2.1.2 Catalyzation of N-nitrosation

As was explained previously (3.5 Alkylating N-nitroso compounds), there are several possible routes of NOC-exposure, including dietary intake and endogenous formation in the gut. Several studies have demonstrated that dietary heme iron, but not inorganic iron and/or meat protein, significantly increases fecal NOC-levels of human volunteers consuming a red meat diet [201-204]. Hence, it appears that heme iron catalyzes NOC-formation upon red meat digestion. More specifically, it has been hypothesized that heme can capture NO (e.g. after release by S-nitrosothiols under alkaline conditions in the small bowel), resulting in the formation of nitrosyl heme. Since nitrosyl heme can act as a nitrosating agent, heme iron could indeed promote endogenous NOC-formation [195].

The link between processed meat consumption and CRC appears to be more pronounced than for fresh red meat consumption. The vast majority of processed meats are produced by curing of red meat like beef, and mainly pork. After curing, the heme iron present is readily nitrosylated. Furthermore, Santarelli et al. documented that heme nitrosylation appears to be essential for the promotion of CRC through processed meat digestion [205]. Nitrosylated heme iron appears to be more reactive and thus also more toxic than non-nitrosylated heme iron [190, 205, 206], possibly explaining the more pronounced association of processed meat consumption with CRC.

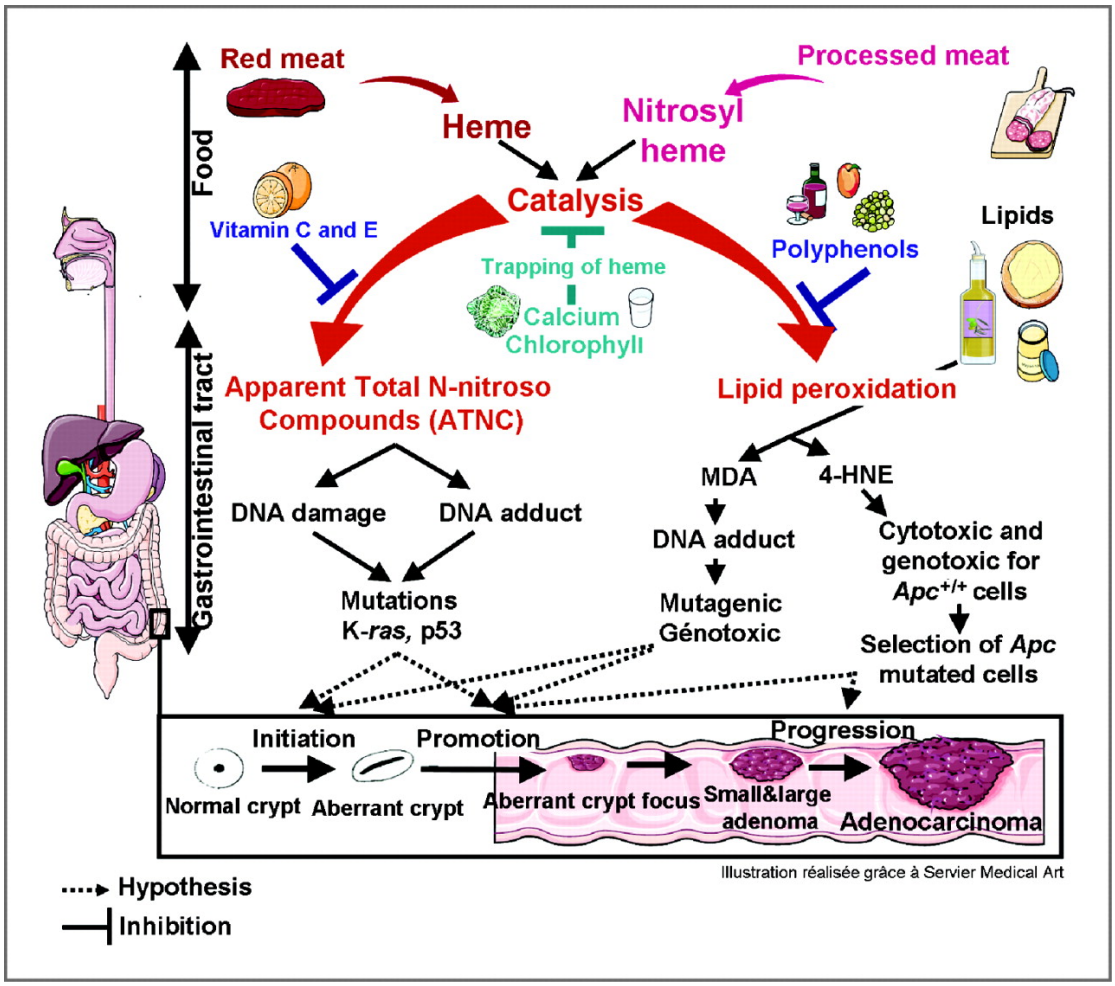

Figure 10. Hypotheses on CRC promotion and inhibition through the diet, including the heme hypothesis, according to Bastide et al. [190].

4.3.2.2 The white meat controversy

The so-called 'white meat controversy' is based on the observation that (1) processed meat consumption has a more pronounced correlation with CRC risk compared to fresh red meat, (2) processed meat is mainly constituted from pork, but (3) the difference in heme iron content between pork and white meat is not very pronounced, questioning the the dose-response effect of heme with regard to CRC. So, although the heme hypothesis has received a lot of scientific support, it appears that the heme hypothesis can only partly explain the link between (processed) meat consumption and an increased risk of CRC.

In light of this, Demeyer, Mertens, De Smet and Ulens [191] recently discussed the potential interfering role of mammalian cell surface sialic acid N-glycolylneuraminic acid (Neu5Gc). Humans cannot produce Neu5Gc, but accumulate Neu5Gc from red meat (lamb, pork and beef) and bovine milk consumption [207]. Since Neu5Gc is a 'foreign' molecule, production of antibodies and chronic inflammation might occur in response [208], hence contributing to the progression of CRC as inflammation is suggested to play a role in CRC development. The intake of milk products is inversely associated with CRC risk, but the synergistic effects of heme and Neu5Gc in (processed) red meat might offer a plausible explanation for the carcinogenic actions of (processed) red meat. Furthermore, the significantly higher levels of calcium in milk compared to processed meat, may further explain the adverse health effects of processed meat consumption, but CRC protective attributes of the consumption of calcium-rich dairy products [12].

4.3.2.3 Heterocyclic amines and polycyclic aromatic hydrocarbons

It was hypothesized that the formation of HCAs and PAHs during high temperature cooking of meat could be the underlying cause of red and processed meat carcinogenicity in the colon and rectum. However, since it has repeatedly been documented that cooked fish and poultry (= white meat) often contain equal or even higher levels of HCAs compared to red meat (e.g. beef and pork) [209], and the consumption of red, but not white meat, increases CRC risk, the formation of HCAs could not solely explain the observed link between red meat consumption and CRC [190, 192]. For PAHs, the cooking method, but not the type of meat seems to be associated with the level of exposure to PAHs. Processing of meat other than cooking, e.g. curing and smoking, could potentially also lead to elevated PAH concentrations. Red and processed meat intake as well as CRC risk could not be confidently linked with exposure to higher levels of PAHs [191, 210], rendering the HCA and PAH hypothesis to fall short at first glance. Nevertheless, the white meat controversy ushers to dig deeper; pointing out that additional mechanisms (besides the direct toxic effects of N-nitrosation and (lipid per)oxidation) might also contribute to red and processed meat associated CRC risk. With regard to PAHs and HCAs, it could be of importance that e.g. red meat induced oxidation processes can induce metabolic activation of PAHs and/or HCAs, an essential step in HCA as well as PAH induced mutagenesis and carcinogenesis (as explained previously; 3.2.3 Polycyclic aromatic hydrocarbons

& heterocyclic amines). For example, it has previously been demonstrated that (a) certain LPOs can induce COX-2 upregulation [211], (b) COX-2 can activate PAHs as well as HCAs [212], and (c) COX-2 expression is linked to CRC development [213]. Hence, new as well as old hypotheses should be given sufficient attention and studied more in-depth.

4.4 DNA adduct analysis to assess the genotoxicity of red (and processed red) meat consumption

The field of DNA adduct research is a highly promising area due to the proposed causal link between the prevalence of certain environmental genotoxins, the formation of DNA adducts and the onset of certain nonhereditary cancer types [42-44]. Furthermore, DNA adduct research does not only enable investigation of genotoxin exposure, uptake, and metabolism but can also provide us with information on the individual rate of DNA repair and individual susceptibility to permanent DNA damage, mutagenesis, and carcinogenesis [39, 41]. Luckily, a multidisciplinary approach and the continuously evolving field of analytical apparatus (will be discussed in "Liquid chromatography coupled to mass spectrometry as the analytical tool of choice") available provide us with the appropriate tools for in-depth DNA adduct research in several specialized areas of research. As such, induction of DNA adduct formation due to e.g. N-nitrosation and oxidation processes *via* red meat consumption represents a highly interesting research topic to further unravel the link between red meat consumption and the development of CRC.

5. LIQUID CHROMATOGRAPHY COUPLED TO MASS SPECTROMETRY AS THE ANALYTICAL TOOL OF CHOICE

5.1 The prominent advantages of mass spectrometry-based DNA adduct analysis

DNA adduct analysis requires very sensitive and highly specific analytical techniques and methodologies. For years, ^{32}P-postlabeling was the most utilized technique, and for some DNA adducts types, a sensitivity of 1 adduct per 10^{10} nucleotides could be achieved [214]. Unfortunately, false positives and artifacts are common when using this approach [215]. Mass spectrometry (MS) based detection techniques on the other hand, enable accurate identification of DNA adducts and can also provide structural information, in which other analytical methods often fall short. Multiple analytical techniques have contributed to the current knowledge on DNA adducts and DNA adduct detection techniques, which have been reviewed extensively [107, 216, 217]. The most important (dis)advantages of different analytical technologies/methodologies for DNA adduct analysis are summarized in table 5. This shows that some of the most commonly applied techniques may offer sufficient sensitivity, but without structural information and vice versa, or that specificity and sample preparation issues exist. In contrast, MS detection excels in specificity and structural identification [216-218].

More than a decade ago, Koc et al. stated that the only disadvantage of MS in the field of DNA adduct analysis was its sensitivity [215]. Over the years, sensitivity has continued to improve [28, 218] as different research groups have focused on optimization of DNA adduct detection methods with MS [99, 219-225], including research into non-manual data mining and sequencing to locate DNA adduction sites [226, 227]. Because of ongoing technical advancements and the use of stable isotope labeled internal standards, MS currently offers a reliable tool to measure low DNA adduct levels with the highest specificity [214-216, 218]. Coupling of MS with LC by means of the electrospray interface (ESI) has enabled analysis of DNA adducts in very complex biological matrixes [228] while avoiding complex and labor-intensive sample preparation with derivatization for the initially envisioned use of GC-couplings [229].

Table 5. The most prominent advantages and disadvantages of different analytical DNA adduct detection methods [216, 217].

Analytical technique	Advantage(s)	Disadvantage(s)
Accelerator MS	Most sensitive method for DNA adduct detection (up to 1 adduct per 10^{12} nucleotides)	Need for administration and incorporation of $^{14}C/^{3}H$-labeled compounds, specialized equipment, limited specificity, lack of structural information
Electron capture detection	Sensitive (< 1 adduct per 10^{8} nucleotides)	Only applicable for electro-chemically active DNA adducts; lack of versatility, poor identification
Fluorescence detection	Easy, robust, low cost	Only applicable for fluorescent DNA adducts; lack of versatility
Immunoassay	Easy, robust, low cost	Need for DNA adduct-specific antibodies, cross-reactivity; lack of specificity
Immunohistochemistry	Easy, robust	Need for DNA adduct-specific antibodies, only semi quantitative, poor identification, limited structural information
Ligase Mediated - Polymerase Chain Reaction	Determination of DNA adduction at sequence level	Low sensitivity (≈ 2.5 adducts per 10^{4} nucleotides), variable specificity
Nuclear Magnetic Resonance	Provides structural information and identification	No quantitation
^{32}P-postlabelling	Sensitive (up to 1 adduct per 10^{10} nucleotides), requires low amounts of DNA (1 - 10 μg), versatile, widely applicable	Limited specificity, no structural information, need for radiolabelling
Radiolabeled binding assay	Easy	No structural information, need for radiolabelling, interference with contaminants, need for high amounts of DNA (0.5 – 3 mg)
GC-MS	Provides structural information and identification, good sensitivity; up to1 adduct per 10^{9} nucleotides	Need for specialized equipment (and internal standards), need for derivatization, induction of artifacts (oxidative DNA damage due to derivatization)
LC-MS	Provides structural information and identification, good sensitivity; up to 1 adduct per 10^{9} nucleotides	Need for specialized equipment (and internal standards)

5.2 Targeted vs. untargeted DNA adduct analysis

An important advantage of MS, in contrast to many other DNA adduct detection methods, is the possibility to detect both "targeted" and "untargeted" DNA adducts by means of full scan MS. Targeted DNA adduct detection (also known as "profiling") refers to the detection of known types of DNA adducts, which implies that the MS system specifically scans for the presence of certain compounds of interest to assess their presence and abundance, while all other molecules in the sample are disregarded completely. On the other hand, untargeted analysis (also known as "fingerprinting"), refers to the detection of all compounds present, even if unknown or deemed irrelevant at the time [230]. The full scan data of biological samples can be searched for the presence of other DNA adduct types (known or unknown) in parallel or retrospectively, providing potentially highly relevant additional information. The targeted detection of DNA adducts accords with the bottom-up approach, whereas the untargeted mapping or fingerprinting of DNA adducts facilitates a top-down approach (figure 11).

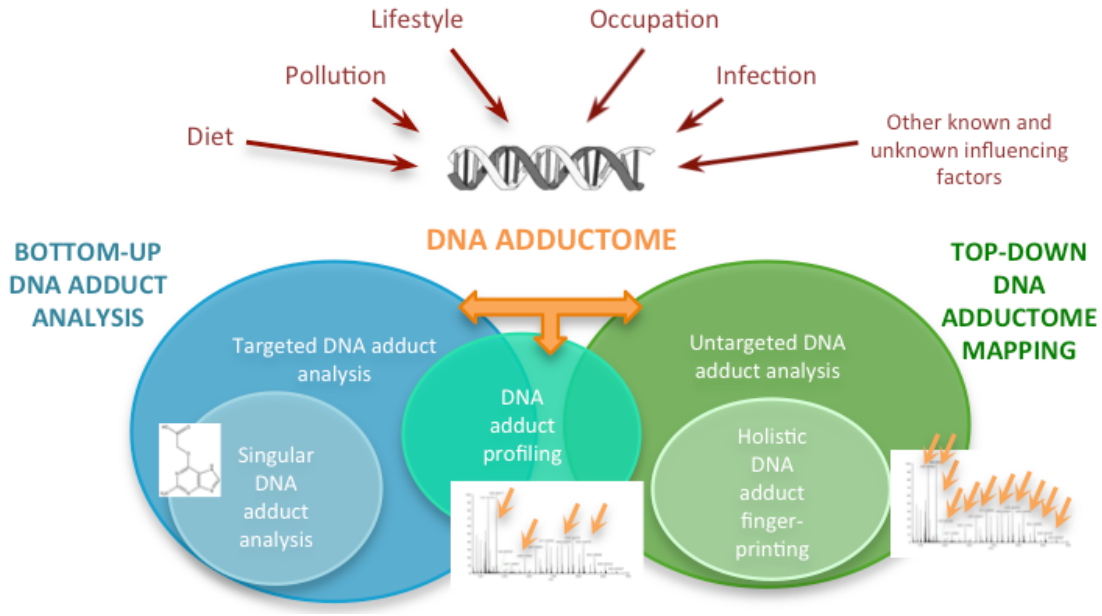

Figure 11. Targeted singular DNA adduct analysis vs. DNA adduct profiling vs. untargeted DNA adduct mapping.

5.3 Practical considerations for (mass-spectrometry-based) DNA adduct research

5.3.1 Internal standards

In recent years, the sensitivity of MS for DNA adduct quantitation purposes has increased significantly. The use of stable isotope labeled internal standards has provided a means to quantitate with high accuracy [215, 217, 218]. An internal standard can be manufactured by replacing one or several atoms in the DNA adduct structure with their ^{2}H, ^{13}C, ^{15}N, or ^{18}O isotopes. Because of the nearly identical chemical characteristics, these types of internal standards are well-suited for correction of variation due to losses during sample handling and preparation, local matrix effects, and possible fluctuations in sensitivity during analysis [215, 218]. In addition, the use of labeled (and unlabeled) (internal) standards facilitates compound identification by enabling comparison of the retention times that were obtained for different compounds.

Unfortunately, appropriate internal standards for DNA adducts are not always easily obtained commercially [216]. Furthermore, there may be some concerns regarding the stability of the labeled internal standards [218]. Some deuterated DNA adduct analogues (e.g., d_2-O^6-CMG) lack chemical stability and are prone to decomposition due to exchange of deuterium for hydrogen [218, 231]. The stability of ^{13}C, ^{15}N, and ^{18}O labeled DNA adducts seems to be less cause for concern, as their degradation likely parallels the breakdown of their unlabeled counterparts [215]. Furthermore, the internal standard should ideally differ in mass by 3 units from that of the compound under investigation. Hence, the use of ^{13}C, ^{15}N and ^{18}O labeled internal standards is the obvious choice.

If there is no appropriate and stable internal standard available at the time, analysis and quantitation by means of an external calibration line offers a possible alternative, although a less accurate approach for DNA adduct quantitation [218]. However, this offers no scope for correction due to sample preparation issues.

5.3.2 DNA adduct stability

Sufficient knowledge on chemical stability of DNA adducts is extremely important for correct interpretation of results [38, 217]. However, it appears that only a limited number of studies have studied the stability of a limited number of adduct types, which was discussed in detail by Himmelstein et al [216]. DNA adduct stability depends on several factors including pH (e.g. M_1dG is not stable under alkaline conditions [232]) and composition of storage buffers (e.g. Tris buffer induces M_1dG instability [233]). Sample matrix (e.g. embedded in liver vs. kidney or other sample types [234]), sample processing (e.g. contamination by RNA can add to N^7-MeG levels [235]), storage temperature (M_1dG and benzo[a]pyrene DNA adducts, among others [233, 236]) and, last but not least, DNA adduct type or chemical composition (e.g. O^6-MeG appears to be more stable than N^7-MeG and N^3-methylguanine [237]) also influence DNA adduct stability. In contrast, it appears that the number of freeze-thaw cycles and long-term storage might not significantly reduce DNA adduct stability [238-240].

In order to avoid incorrect interpretation and loss of results, sample handling and storage should be considered in a case-by-case manner and executed carefully and consistently. To improve knowledge on DNA adduct stability, researchers should opt to conduct more DNA adduct stability studies focusing on different DNA adduct types, sample handling and storage conditions; e.g. taking the use of certain DNA buffers and the optimum storage temperature into account. In the meantime, DNA should preferably be stored at -80°C, while evaporation to dryness may prevent early decomposition of DNA adducts [216].

5.3.3 Sample preparation

Sample preparation is considered to be one of the most critical steps in analytical chemistry in general, but in particular for DNA adduct quantitation since DNA adducts are embedded in a complex matrix of abundantly present unmodified DNA building blocks [215]. To enable the simultaneous detection of a multitude of DNA adducts, sample preparation should be kept to a minimum, as extensive sample preparation procedures may induce artifacts (e.g. formation of oxo-dG during sample preparation), loss of sample and relevant information (due to, e.g., instability issues). In addition, sample preparation (cleanup and enrichment) can be quite time consuming and labor intensive. However, sufficient release of DNA adducts from the DNA

sequence (DNA hydrolysis efficiency), enrichment and removal of unwanted matrix constituents are indispensable [215, 216, 218].

DNA adducts can be detected individually as adducted nucleotides, nucleosides or nucleobases upon DNA hydrolysis, DNA adduct extraction and enrichment. The choice of measuring either adducted oligonucleotides, nucleotides, nucleosides or nucleobases greatly influences sample separation needs. Analysis of (oligo-)nucleotides is least common and requires enzymatic digestion of DNA. Likewise, analysis of nucleosides requires enzymatic digestion and is the most common method of sample preparation for mass spectrometry-based DNA adduct analyses. Thermally labile modified nucleobases can be released by means of thermal hydrolysis. More stable adducted nucleobases can be retrieved with thermal hydrolysis at high temperature or strong acid hydrolysis. By combining acid and thermal hydrolysis, both altered and unaltered nucleobases are cleaved from the DNA sequence [218]. Nonetheless, Kato et al. found that a single approach may not release all adducts, and had to employ two different enzymatic hydrolysis methods to prepare their samples, resulting in a doubled workload [241]. These procedures are lengthy and have multiple steps that may cause changes to the DNA and the adduct profile, which must be thoroughly investigated during method development.

To improve the sensitivity, sample cleanup, and enrichment upon DNA hydrolysis or digestion are highly recommended [214, 216]. The envisioned removal of unmodified DNA building blocks and interfering contaminants (e.g. highly polar compounds that interfere with ionization) is required to minimize signal and ionization suppression [215, 218]. Frequently utilized on- or offline techniques for DNA adduct enrichment include immunoaffinity column purification, HPLC column switching, and solid phase extraction [214, 215, 218]. However, care must be taken with selection of the appropriate stationary phases and elution buffers to avoid degradation/loss of the adducts. In addition, immunoaffinity column purification can only be implemented prior to targeted analysis due to the specificity of the antibodies in use [242]. Therefore, this particular technique is only suited for targeted DNA adduct analyses but not DNA adductomics.

5.3.4 Study design

The choice of an appropriate study design is considered to be one of the most important factors in DNA adduct studies. The exposome is very complex, dynamic, and continuously changing [26]. Therefore, measurement of the exposome or DNA adductome at one isolated moment in time will not answer all related research questions. Thus, assessment of individual exposure requires longitudinal studies [25, 38]. According to Wild, full characterization of the individual exposome requires an extensive number of sequential measures throughout a lifetime, or at the least, a smaller number of measures to assess exposures over a series of extended periods [26]. In addition, exposomics studies should also consider the interfering influence of other very important factors like sample handling, fixation, storage and tracking, lack of tissue homogeneity, differences in individual susceptibility, and genetic polymorphisms [38, 243]. Consequently, only well throughout long-term and large-scale (e.g. many individuals, appropriate controls and different tissue types) studies will enable correct and thorough assessment of DNA adduct profile.

5.3.5 Surrogate vs. target tissue

Ideally, DNA adduct formation should be monitored in the considered target tissue. A technical and ethical difficulty is that target tissue is not always easily obtained and/or available. A possible solution to that problem is the use of appropriate surrogate tissues like blood, urine, and exfoliated (e.g. buccal or gastrointestinal epithelial) cells, provided that DNA adduct levels in target and surrogate tissue are distinctly related and a sufficient amount of DNA can be collected. Typically, procedures use an initial amount of 100 μg of DNA although some require less; e.g. the most recent method by Kanaly et al. used 15 μg of DNA per injection [244]. If no data on correlation of particular DNA adducts in surrogate vs. target tissue are available, correlation studies should be performed during or prior to biomonitoring studies. Researchers must also consider the possibility that a certain type of surrogate tissue may be more appropriate than others or that a well-suited surrogate tissue simply does not exist [38, 245]. For example, although Wiencke et al. [246] were able to demonstrate the use of mononuclear blood cells as an appropriate surrogate tissue for lung tissue to study tobacco-associated DNA adduct formation, Kriek and coworkers were unable to correlate PAH-DNA adduct levels in white blood cells and lung tissue in lung cancer patients [247].

5.3.6 Method validation

Over the past 30 years, several papers have reported the development of new methods for the detection of single or plural DNA adducts in different matrixes. The use of analytical methods for DNA adduct detection and quantification in biomonitoring studies necessitates thorough evaluation of reliability and fit-for-purpose. This requires assured specificity, accuracy, precision and sensitivity, acceptable recovery and reproducibility, information about the assay and compound stability, and the assessment of detection and quantification limits [248]. Moreover, intra- and inter-laboratory variability need to be assessed and properly addressed [216]. However, at present, detailed and specific guidelines concerning the validation and interpretation of validation parameters of an analytical method for the detection of DNA adducts or possible biomarkers do not exist.

Since biomarkers could provide interesting opportunities for the pharmaceutical industry, both the U.S. Food and Drug Administration (FDA) and European Medicines Agency (EMA) are aware of the urgency to establish proper validation guidelines for biomarker assays. In consequence, suitable guidelines are to be published as soon as possible [248, 249]. In 2013, the FDA published draft guidance for industry on bioanalytical method validation. In this draft guidance, the agency stated the following: "The accuracy, precision, selectivity, range, reproducibility, and stability of a biomarker assay are important characteristics that define the method. The approach used for pharmacokinetic assays should be the starting point for validation of biomarker assays, although FDA realizes that some characteristics may not apply or that different considerations may need to be addressed" [248]. In agreement, the EMA published a concept paper on good genomics biomarker practices in 2014 [249], which acknowledged and documented the need for guidance concerning choice and proper use of technology and methodology for genomic biomarker analysis in a clinical setting. Since DNA adducts are regarded as biomarkers of exposure and (possible) biomarkers of effect, the future FDA and EMA guidelines could provide a basis for validation of DNA adduct detection methods as well.

6. SPECIFIC FOCUS AND AIM OF THIS STUDY

This PhD project envisioned to further unravel the possible causative role of red meat consumption in colorectal carcinogenesis *via* chemically induced DNA adduct formation.

At the very start of this PhD, the possible cyto- and genotoxic effects of red *vs.* white meat were studied by means of the *in vitro* digestion of beef, pork and chicken. More specifically, the effect of meat type and heme content on cytotoxicity, lipid peroxidation, and the formation of 2 specific NOC-related DNA adducts (i.e. O^6-CMG and O^6-MeG) were assessed using a targeted approach, all of which is described in **Chapter II**.

To allow more in-depth assessment of the genotoxic effects of red meat consumption and digestion, the need for an untargeted 'omics' approach presented itself. Therefore, to be able to extensively investigate alkylation and (lipid per)oxidation related DNA adduct formation in relation to the NOC hypothesis as well as the lipid peroxidation hypothesis, a DNA adductomics methodology was developed and validated successfully. The optimization as well as a demonstration of the *in vitro* and *in vivo* application of the new methodology is described in **Chapter III**.

Chapter IV reports the use of the newly developed DNA adductomics methodology to map the DNA adductome of red vs. white meat digests that were obtained by means of the *in vitro* digestion of chicken and beef. The effect of the addition of calcium (to meat preparations) on DNA adduct formation was investigated as well.

In **Chapter V**, red *vs.* white meat genotoxicity, in the form of DNA adduct formation, was investigated on a much larger scale. In addition, the interfering role of myoglobin, carnitine or lysine was investigated to further unravel and mechanistically support the underlying mechanisms of red meat toxicity.

The hypothesized genotoxicity of red meat consumption was also assessed *in vivo*, i.e. **Chapter VI** describes a rat feeding trial, during which 24 Sprague-Dawley rats received a diet with beef or chicken. Furthermore, to assess the interfering role of dietary fat, some rats were fed a chicken or beef diet supplemented with lard. After the 14-day feeding trial, shifts in the DNA adductome of liver, duodenum and colon were assessed to investigate the *in vivo* formation of DNA adducts due to red meat and/or lard digestion.

Finally, in **Chapter VII**, the main findings and conclusions from this PhD project are recapitulated, integrated and discussed comprehensively. The future needs, challenges and perspectives are addressed as well.

Throughout this dissertation, the following research questions will be addressed:

- Does red meat digestion promote lipid peroxidation?
- Does red meat digestion stimulate the formation of N-nitroso compounds?
- Can red meat digestion interfere with cell proliferation and viability?
- Does red meat digestion give rise to the (increased) formation of certain types of DNA adducts?
- How does red meat digestion alter the DNA adductome?
- Does the supplementation of calcium alter the DNA adductome?
- Does the addition of lard (to a meat preparation) alter the DNA adductome?
- Does myoglobin addition alter the DNA adductome?

REFERENCES

[1] J. Ferlay, I. Soerjomataram, M. Ervik, R. Dikshit, S. Eser, C. Mather, M. Rebelo, D.M. Parkin, D. Forman, F. Bray, GLOBOCAN 2012 v1.0, Cancer Incidence and Mortality Worldwide, GLOBOCAN 2012; International Agency for Research on Cancer. Available from: http://globocan.iarc.fr, (2013).
[2] R. John, H. Ross, The global economic cost of cancer, American Cancer Society & LIVESTRONG organisation Report, (2010).
[3] Cancer Research UK, http://www.cancerresearchuk.org/health-professional/cancer-statistics/survival, accessed November, 2016.
[4] J.H. Breasted, editor, The Classics of Surgery Library. The Edwin Smith Surgical Papyrus, The University Chicago Press, Division of Gryphon Editions, Ltd. Birmingham (AB). Frontispiece., Chicago, Illinois, 1930, special edition 1984.
[5] Homer, Iliad, Rouse WHD, Transl. New York: New American Library, New York, 1966.
[6] R.H. Young, D.N. Louis, The Warrens and other pioneering clinician pathologists of the Massachusetts General Hospital during its early years: an appreciation on the 200th anniversary of the hospital founding, Mod Pathol, 24 (2011) 1285-1294.
[7] R. Lakhtakia, R.F. Chinoy, A Brief History of Breast Cancer: Part II - Evolution of surgical pathology, Sultan Qaboos University Medical Journal, 14 (2014) e319-e322.
[8] R. Lakhtakia, A Brief History of Breast Cancer: Part I: Surgical domination reinvented, Sultan Qaboos University Medical Journal, 14 (2014) e166-e169.
[9] H.W. Herr, Percivall Pott, the environment and cancer, BJU Int, 108 (2011) 479-481.
[10] P. Anand, A.B. Kunnumakara, C. Sundaram, K.B. Harikumar, S.T. Tharakan, O.S. Lai, B. Sung, B.B. Aggarwal, Cancer is a Preventable Disease that Requires Major Lifestyle Changes, Pharmaceutical Research, 25 (2008) 2097-2116.
[11] S.M. Rappaport, Discovering environmental causes of disease, Journal of epidemiology and community health, 66 (2012) 99-102.
[12] B. Stewart, C.P. Wild, editors, World Cancer Report 2014 International Agency for Research on Cancer, WHO2014.
[13] A. Weston, C.C. Harris, Multistage Carcinogenesis, Hamilton, BC Decker2003.
[14] W. Troll, R. Wiesner, The role of oxygen radicals as a possible mechanism of tumor promotion, Annu Rev Pharmacol Toxicol, 25 (1985) 509-528.
[15] H.C. Pitot, T. Goldsworthy, S. Moran, The natural history of carcinogenesis: implications of experimental carcinogenesis in the genesis of human cancer, J Supramol Struct Cell Biochem, 17 (1981) 133-146.
[16] h.w.h.h.e.d.-a.-c.t.-f.-c.-p.-n.-w. Harvard Health Publications, accessed February, 2017.
[17] T.J. Key, N.E. Allen, E.A. Spencer, R.C. Travis, The effect of diet on risk of cancer, The Lancet, 360 (2002) 861-868.
[18] C. Ioannides, M. Delaforge, D.V. Parke, Safrole: Its metabolism, carcinogenicity and interactions with cytochrome P-450, Food and Cosmetics Toxicology, 19 (1981) 657-666.
[19] B.L. Smith, A.A. Seawright, Bracken fern (Pteridium spp.) carcinogenicity and human health--a brief review, Natural toxins, 3 (1995) 1-5.
[20] A.G. Marroquin-Cardona, N.M. Johnson, T.D. Phillips, A.W. Hayes, Mycotoxins in a changing global environment--a review, Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association, 69 (2014) 220-230.
[21] R.J. Turesky, Formation and biochemistry of carcinogenic heterocyclic aromatic amines in cooked meats, Toxicology letters, 168 (2007) 219-227.
[22] S.P. Claus, H. Guillou, S. Ellero-Simatos, The gut microbiota: a major player in the toxicity of environmental pollutants?, Npj Biofilms And Microbiomes, 2 (2016) 16003.

[23] F.P. Perera, Environment and Cancer: Who Are Susceptible?, Science (New York, N.Y.), 278 (1997) 1068-1073.
[24] C.P. Wild, Complementing the Genome with an "Exposome": The Outstanding Challenge of Environmental Exposure Measurement in Molecular Epidemiology, Cancer Epidemiology Biomarkers & Prevention, 14 (2005) 1847-1850.
[25] S.S. Coughlin, Toward a road map for global -omics: a primer on -omic technologies, American journal of epidemiology, 180 (2014) 1188-1195.
[26] C.P. Wild, The exposome: from concept to utility, International Journal of Epidemiology, 41 (2012) 24-32.
[27] M.C. Poirier, R.M. Santella, A. Weston, Carcinogen macromolecular adducts and their measurement, Carcinogenesis, 21 (2000) 353-359.
[28] P.B. Farmer, K. Brown, E. Tompkins, V.L. Emms, D.J. Jones, R. Singh, D.H. Phillips, DNA adducts: mass spectrometry methods and future prospects, Toxicology and applied pharmacology, 207 (2005) 293-301.
[29] R. Colin Garner, The role of DNA adducts in chemical carcinogenesis, Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 402 (1998) 67-75.
[30] R. De Bont, N. van Larebeke, Endogenous DNA damage in humans: a review of quantitative data, Mutagenesis, 19 (2004) 169-185.
[31] G.N. Wogan, S.S. Hecht, J.S. Felton, A.H. Conney, L.A. Loeb, Environmental and chemical carcinogenesis, Seminars in cancer biology, 14 (2004) 473-486.
[32] J.S. Wang, J.D. Groopman, DNA damage by mycotoxins, Mutation research, 424 (1999) 167-181.
[33] A. Besaratinia, G.P. Pfeifer, DNA adduction and mutagenic properties of acrylamide, Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 580 (2005) 31-40.
[34] R.J. Turesky, P. Vouros, Formation and analysis of heterocyclic aromatic amine–DNA adducts in vitro and in vivo, Journal of Chromatography B, 802 (2004) 155-166.
[35] J.K. Wiencke, DNA adduct burden and tobacco carcinogenesis, Oncogene, 21 (2002) 7376-7391.
[36] K. Hemminki, E. Grzybowska, M. Chorazy, K. Twardowska-Saucha, J.W. Sroczynski, K.L. Putman, K. Randerath, D.H. Phillips, A. Hewer, R.M. Santella, et al., DNA adducts in human environmentally exposed to aromatic compounds in an industrial area of Poland, Carcinogenesis, 11 (1990) 1229-1231.
[37] H.S. Yu, T. Oyama, T. Isse, K. Kitagawa, T.T. Pham, M. Tanaka, T. Kawamoto, Formation of acetaldehyde-derived DNA adducts due to alcohol exposure, Chem Biol Interact, 188 (2010) 367-375.
[38] A. Rundle, Carcinogen-DNA adducts as a biomarker for cancer risk, Mutation research, 600 (2006) 23-36.
[39] F.P. Perera, I.B. Weinstein, Molecular epidemiology: recent advances and future directions, Carcinogenesis, 21 (2000) 517-524.
[40] S. Mollerup, D. Ryberg, A. Hewer, D.H. Phillips, A. Haugen, Sex differences in lung CYP1A1 expression and DNA adduct levels among lung cancer patients, Cancer Res, 59 (1999) 3317-3320.
[41] X.D. Long, H.D. Huang, X.Y. Huang, J.G. Yao, Q. Xia, XPC codon 939 polymorphism is associated with susceptibility to DNA damage induced by aflatoxin B1 exposure, International journal of clinical and experimental medicine, 8 (2015) 1197-1204.
[42] G.S. Qian, R.K. Ross, M.C. Yu, J.M. Yuan, Y.T. Gao, B.E. Henderson, G.N. Wogan, J.D. Groopman, A follow-up study of urinary markers of aflatoxin exposure and liver cancer risk in Shanghai, People's Republic of China, Cancer epidemiology, biomarkers & prevention : a

publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology, 3 (1994) 3-10.
[43] J. Angerer, U. Ewers, M. Wilhelm, Human biomonitoring: State of the art, International Journal of Hygiene and Environmental Health, 210 (2007) 201-228.
[44] B. Jelakovic, S. Karanovic, I. Vukovic-Lela, F. Miller, K.L. Edwards, J. Nikolic, K. Tomic, N. Slade, B. Brdar, R.J. Turesky, Z. Stipancic, D. Dittrich, A.P. Grollman, K.G. Dickman, Aristolactam-DNA adducts are a biomarker of environmental exposure to aristolochic acid, Kidney international, 81 (2012) 559-567.
[45] IARC, IARC Monographs on the Evaluation of Carcinogenic Risks to Humans: Some Naturally Occurring Substances: Food Items and Constituents, Heterocyclic Aromatic Amines and Mycotoxins., IARC Scientific Publications, 56 (1993).
[46] R.K. Ross, J.M. Yuan, M.C. Yu, G.N. Wogan, G.S. Qian, J.T. Tu, J.D. Groopman, Y.T. Gao, B.E. Henderson, Urinary aflatoxin biomarkers and risk of hepatocellular carcinoma, Lancet, 339 (1992) 943-946.
[47] A. Pfohl-Leszkowicz, K. Chakor, E.E. Creppy, G. Dirheimer, DNA adduct formation in mice treated with ochratoxin A, IARC scientific publications, (1991) 245-253.
[48] A. Pfohl-Leszkowicz, L. Chekir-Ghedira, H. Bacha, Genotoxicity of zearalenone, an estrogenic mycotoxin: DNA adduct formation in female mouse tissues, Carcinogenesis, 16 (1995) 2315-2320.
[49] I.R. McConnell, R.C. Garner, DNA adducts of aflatoxins, sterigmatocystin and other mycotoxins, IARC Sci Publ, (1994) 49-55.
[50] V.R. Preedy, R.R. Watson, Reviews in Food and Nutrition Toxicity, 2005.
[51] R.F. Omar, B.B. Hasinoff, F. Mejilla, A.D. Rahimtula, Mechanism of ochratoxin A stimulated lipid peroxidation, Biochemical pharmacology, 40 (1990) 1183-1191.
[52] H. Wiedenfeld, J. Edgar, Toxicity of pyrrolizidine alkaloids to humans and ruminants, Phytochem Rev, 10 (2011) 137-151.
[53] J.A. Edgar, R.J. Molyneux, S.M. Colegate, Pyrrolizidine Alkaloids: Potential Role in the Etiology of Cancers, Pulmonary Hypertension, Congenital Anomalies, and Liver Disease, Chemical research in toxicology, (2014).
[54] Y. Zhao, Q. Xia, G. Gamboa da Costa, H. Yu, L. Cai, P.P. Fu, Full Structure Assignments of Pyrrolizidine Alkaloid DNA Adducts and Mechanism of Tumor Initiation, Chemical research in toxicology, 25 (2012) 1985-1996.
[55] Q. Xia, L. Ma, X. He, L. Cai, P.P. Fu, 7-glutathione pyrrole adduct: a potential DNA reactive metabolite of pyrrolizidine alkaloids, Chemical research in toxicology, 28 (2015) 615-620.
[56] D.M. Potter, M.S. Baird, Carcinogenic effects of ptaquiloside in bracken fern and related compounds, British journal of cancer, 83 (2000) 914-920.
[57] G.R. Fenwick, Bracken (Pteridium aquilinum)—toxic effects and toxic constituents, Journal of the Science of Food and Agriculture, 46 (1989) 147-173.
[58] O.P. Galpin, C.J. Whitaker, R. Whitaker, J.Y. Kassab, Gastric cancer in Gwynedd. Possible links with bracken, British journal of cancer, 61 (1990) 737-740.
[59] W.H. Hodge, Fern Foods of Japan and the Problem of Toxicity, American Fern Journal, 63 (1973) 77-80.
[60] M.E. Alonso-Amelot, M. Avendano, Possible association between gastric cancer and bracken fern in Venezuela: an epidemiologic study, International journal of cancer. Journal international du cancer, 91 (2001) 252-259.
[61] R.N. Freitas, P.J. O'Connor, A.S. Prakash, M. Shahin, A.C. Povey, Bracken (Pteridium aquilinum)-induced DNA adducts in mouse tissues are different from the adduct induced by the activated form of the Bracken carcinogen ptaquiloside, Biochemical and biophysical research communications, 281 (2001) 589-594.

[62] A.S. Prakash, T.N. Pereira, B.L. Smith, G. Shaw, A.A. Seawright, Mechanism of bracken fern carcinogenesis: evidence for H-ras activation via initial adenine alkylation by ptaquiloside, Natural toxins, 4 (1996) 221-227.
[63] M. Shahin, M.R. Moore, S. Worrall, B.L. Smith, A.A. Seawright, A.S. Prakash, H-ras activation is an early event in the ptaquiloside-induced carcinogenesis: comparison of acute and chronic toxicity in rats, Biochemical and biophysical research communications, 250 (1998) 491-497.
[64] M. Whiting, Toxicity of cycads, Econ Bot, 17 (1963) 270-302.
[65] D.L. Jones, Cycads of the World: Ancient Plants in Today's Landscape, 2002, pp. 456.
[66] F. Esclaire, G. Kisby, P. Spencer, J. Milne, M. Lesort, J. Hugon, The Guam cycad toxin methylazoxymethanol damages neuronal DNA and modulates tau mRNA expression and excitotoxicity, Experimental neurology, 155 (1999) 11-21.
[67] A. Moretti, S. Sabato, G.S. Gigliano, Taxonomic significance of methylazoxymethanol glycosides in the cycads, Phytochemistry, 22 (1983) 115-117.
[68] I. Hirono, Natural carcinogenic products of plant origin, Critical reviews in toxicology, 8 (1981) 235-277.
[69] Y.T. Chung, C.L. Chen, C.C. Wu, S.A. Chan, C.W. Chi, T.Y. Liu, Safrole-DNA adduct in hepatocellular carcinoma associated with betel quid chewing, Toxicology letters, 183 (2008) 21-27.
[70] C.L. Chen, C.W. Chi, K.W. Chang, T.Y. Liu, Safrole-like DNA adducts in oral tissue from oral cancer patients with a betel quid chewing history, Carcinogenesis, 20 (1999) 2331-2334.
[71] J.M. Lee, T.Y. Liu, D.C. Wu, H.C. Tang, J. Leh, M.T. Wu, H.H. Hsu, P.M. Huang, J.S. Chen, C.J. Lee, Y.C. Lee, Safrole-DNA adducts in tissues from esophageal cancer patients: clues to areca-related esophageal carcinogenesis, Mutation research, 565 (2005) 121-128.
[72] T.Y. Liu, C.C. Chen, C.L. Chen, C.W. Chi, Safrole-induced oxidative damage in the liver of Sprague-Dawley rats, Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association, 37 (1999) 697-702.
[73] S.C.o.F. European Commission, Opinion of the Scientific Committee on Food on the safety of the presence of safrole (1-allyl-3,4-methylene dioxy benzene) in flavourings and other food ingredients with flavouring properties, 2002.
[74] E.C. Hagan, P.M. Jenner, W.I. Jones, O.G. Fitzhugh, E.L. Long, J.G. Brouwer, W.K. Webb, Toxic properties of compounds related to safrole, Toxicology and applied pharmacology, 7 (1965) 18-24.
[75] I.M. Rietjens, M.G. Boersma, H. van der Woude, S.M. Jeurissen, M.E. Schutte, G.M. Alink, Flavonoids and alkenylbenzenes: mechanisms of mutagenic action and carcinogenic risk, Mutation research, 574 (2005) 124-138.
[76] S.C.o.F. European Commission, Opinion of the Scientific Committee on Food on Estragole (1-Allyl-4-methoxybenzene), (2001).
[77] A. Paini, A. Punt, G. Scholz, E. Gremaud, B. Spenkelink, G. Alink, B. Schilter, P.J. van Bladeren, I.M.C.M. Rietjens, In vivo validation of DNA adduct formation by estragole in rats predicted by physiologically based biodynamic modelling, Mutagenesis, 27 (2012) 653-663.
[78] R.L. Smith, T.B. Adams, J. Doull, V.J. Feron, J.I. Goodman, L.J. Marnett, P.S. Portoghese, W.J. Waddell, B.M. Wagner, A.E. Rogers, J. Caldwell, I.G. Sipes, Safety assessment of allylalkoxybenzene derivatives used as flavouring substances - methyl eugenol and estragole, Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association, 40 (2002) 851-870.
[79] G.D. Zhou, B. Moorthy, J. Bi, K.C. Donnelly, K. Randerath, DNA adducts from alkoxyallylbenzene herb and spice constituents in cultured human (HepG2) cells, Environmental and molecular mutagenesis, 48 (2007) 715-721.

[80] V.M. Arlt, M. Stiborova, H.H. Schmeiser, Aristolochic acid as a probable human cancer hazard in herbal remedies: a review, Mutagenesis, 17 (2002) 265-277.
[81] U. Mengs, W. Lang, J.A. Poch, The carcinogenic action of aristolochic acid in rats, Arch Toxicol, 51 (1982) 107-119.
[82] U. Mengs, Tumour induction in mice following exposure to aristolochic acid, Arch Toxicol, 61 (1988) 504-505.
[83] U. Mengs, Acute toxicity of aristolochic acid in rodents, Arch Toxicol, 59 (1987) 328-331.
[84] F.D. Debelle, J.L. Vanherweghem, J.L. Nortier, Aristolochic acid nephropathy: a worldwide problem, Kidney international, 74 (2008) 158-169.
[85] V.M. Arlt, H.H. Schmeiser, G.P. Pfeifer, Sequence-specific detection of aristolochic acid–DNA adducts in the human p53 gene by terminal transferase-dependent PCR, Carcinogenesis, 22 (2001) 133-140.
[86] H.H. Schmeiser, C.A. Bieler, M. Wiessler, C. van Ypersele de Strihou, J.-P. Cosyns, Detection of DNA Adducts Formed by Aristolochic Acid in Renal Tissue from Patients with Chinese Herbs Nephropathy, Cancer Research, 56 (1996) 2025-2028.
[87] G.M. Lord, T. Cook, V.M. Arlt, H.H. Schmeiser, G. Williams, C.D. Pusey, Urothelial malignant disease and Chinese herbal nephropathy, Lancet, 358 (2001) 1515-1516.
[88] G.M. Lord, M. Hollstein, V.M. Arlt, C. Roufosse, C.D. Pusey, T. Cook, H.H. Schmeiser, DNA adducts and p53 mutations in a patient with aristolochic acid-associated nephropathy, Am J Kidney Dis, 43 (2004) e11-17.
[89] C.-H. Chen, K.G. Dickman, M. Moriya, J. Zavadil, V.S. Sidorenko, K.L. Edwards, D.V. Gnatenko, L. Wu, R.J. Turesky, X.-R. Wu, Y.-S. Pu, A.P. Grollman, Aristolochic acid-associated urothelial cancer in Taiwan, Proceedings of the National Academy of Sciences, 109 (2012) 8241-8246.
[90] M.Z. Hossain, S.F. Gilbert, K. Patel, S. Ghosh, A.K. Bhunia, S.E. Kern, Biological clues to potent DNA-damaging activities in food and flavoring, Food and Chemical Toxicology, 55 (2013) 557-567.
[91] A. Nkondjock, Coffee consumption and the risk of cancer: an overview, Cancer letters, 277 (2009) 121-125.
[92] E.G. de Mejia, M.V. Ramirez-Mares, S. Puangpraphant, Bioactive components of tea: Cancer, inflammation and behavior, Brain, Behavior, and Immunity, 23 (2009) 721-731.
[93] G. Souid-Mensi, S. Moukha, T.A. Mobio, K. Maaroufi, E.E. Creppy, The cytotoxicity and genotoxicity of okadaic acid are cell-line dependent, Toxicon, 51 (2008) 1338-1344.
[94] A. Traoré, S. Ruiz, I. Baudrimont, A. Sanni, S.D. Dano, P. Guarigues, J.F. Narbonne, E.E. Creppy, Combined effects of okadaic acid and cadmium on lipid peroxidation and DNA bases modifications (m5dC and 8-(OH)-dG) in Caco-2 cells, Arch Toxicol, 74 (2000) 79-84.
[95] F.F. Radwan, J.S. Ramsdell, Brevetoxin forms covalent DNA adducts in rat lung following intratracheal exposure, Environmental health perspectives, 116 (2008) 930-936.
[96] T. Çavaş, S. Könen, In vivo genotoxicity testing of the amnesic shellfish poison (domoic acid) in piscine erythrocytes using the micronucleus test and the comet assay, Aquatic Toxicology, 90 (2008) 154-159.
[97] S.C.o.F. European Commission, Opinion of the Scientific Committee on Food on Isosafrole, (2003).
[98] S.C.o.F. European Commission, Opinion of the Scientific Committee on Food on Methyleugenol (4-Allyl-1,2-dimethoxybenzene), (2001).
[99] G. Gamboa da Costa, M.I. Churchwell, L.P. Hamilton, L.S. Von Tungeln, F.A. Beland, M.M. Marques, D.R. Doerge, DNA Adduct Formation from Acrylamide via Conversion To Glycidamide in Adult and Neonatal Mice, Chemical research in toxicology, 16 (2003) 1328-1337.

[100] IARC, IARC Monographs on the Evaluation of Carcinogenic Risks to Humans; Some Industrial Chemicals, Acrylamide, IARC Scientific Publications, 60 (1994).
[101] I. Manière, T. Godard, D.R. Doerge, M.I. Churchwell, M. Guffroy, M. Laurentie, J.-M. Poul, DNA damage and DNA adduct formation in rat tissues following oral administration of acrylamide, Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 580 (2005) 119-129.
[102] C. Pelucchi, C. Bosetti, C. Galeone, C. La Vecchia, Dietary acrylamide and cancer risk: an updated meta-analysis, International journal of cancer. Journal international du cancer, 136 (2015) 2912-2922.
[103] S. Hasnip, C. Crews, L. Castle, Some factors affecting the formation of furan in heated foods, Food additives and contaminants, 23 (2006) 219-227.
[104] V.A. Yaylayan, Precursors, Formation and Determination of Furan in Food, J. Verbr. Lebensm., 1 (2006) 5-9.
[105] M.C. Byrns, C.C. Vu, J.W. Neidigh, J.L. Abad, R.A. Jones, L.A. Peterson, Detection of DNA adducts derived from the reactive metabolite of furan, cis-2-butene-1,4-dial, Chemical research in toxicology, 19 (2006) 414-420.
[106] B.H. Monien, K. Herrmann, S. Florian, H. Glatt, Metabolic activation of furfuryl alcohol: formation of 2-methylfuranyl DNA adducts in Salmonella typhimurium strains expressing human sulfotransferase 1A1 and in FVB/N mice, Carcinogenesis, 32 (2011) 1533-1539.
[107] D.H. Phillips, DNA adducts as markers of exposure and risk, Mutation research, 577 (2005) 284-292.
[108] E. Kriek, M. Rojas, K. Alexandrov, H. Bartsch, Polycyclic aromatic hydrocarbon-DNA adducts in humans: relevance as biomarkers for exposure and cancer risk, Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 400 (1998) 215-231.
[109] N. Rothman, A. Correa-Villasenor, D.P. Ford, M.C. Poirier, R. Haas, J.A. Hansen, T. O'Toole, P.T. Strickland, Contribution of occupation and diet to white blood cell polycyclic aromatic hydrocarbon-DNA adducts in wildland firefighters, Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology, 2 (1993) 341-347.
[110] L. Vanhaecke, Impact of the human intestinal microbiota on the metabolism and toxic properties of the meat contaminant 2-amino-1-methyl-6-phenylimidazo[4,5- b]pyridine, PhD Thesis, 2008.
[111] D.H. Kang, N. Rothman, M.C. Poirier, A. Greenberg, C.H. Hsu, B.S. Schwartz, M.E. Baser, J.D. Groopman, A. Weston, P.T. Strickland, Interindividual differences in the concentration of 1-hydroxypyrene-glucuronide in urine and polycyclic aromatic hydrocarbon-DNA adducts in peripheral white blood cells after charbroiled beef consumption, Carcinogenesis, 16 (1995) 1079-1085.
[112] B.H. Monien, W. Engst, G. Barknowitz, A. Seidel, H. Glatt, Mutagenicity of 5-hydroxymethylfurfural in V79 cells expressing human SULT1A1: identification and mass spectrometric quantification of DNA adducts formed, Chemical research in toxicology, 25 (2012) 1484-1492.
[113] E.G. Rogan, P.D. Devanesan, N.V. RamaKrishna, S. Higginbotham, N.S. Padmavathi, K. Chapman, E.L. Cavalieri, H. Jeong, R. Jankowiak, G.J. Small, Identification and quantitation of benzo[a]pyrene-DNA adducts formed in mouse skin, Chemical research in toxicology, 6 (1993) 356-363.
[114] IARC, IARC Monographs on the Evaluation of Carcinogenic Risks to Humans: Alcohol Drinking., IARC Scientific Publications, (1988) 153-246.
[115] R. Baan, K. Straif, Y. Grosse, B. Secretan, F. El Ghissassi, V. Bouvard, A. Altieri, V. Cogliano, Carcinogenicity of alcoholic beverages, The Lancet. Oncology, 8 (2007) 292-293.

[116] IARC, IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. Re-evaluation of Some Organic Chemicals, Hydrazine and Hydrogen peroxide (part two). IARC Scientific Publications, (1999) 319-335.
[117] J.L. Fang, C.E. Vaca, Detection of DNA adducts of acetaldehyde in peripheral white blood cells of alcohol abusers, Carcinogenesis, 18 (1997) 627-632.
[118] C.E. Vaca, J.A. Nilsson, J.L. Fang, R.C. Grafstrom, Formation of DNA adducts in human buccal epithelial cells exposed to acetaldehyde and methylglyoxal in vitro, Chem Biol Interact, 108 (1998) 197-208.
[119] M. Wang, E.J. McIntee, G. Cheng, Y. Shi, P.W. Villalta, S.S. Hecht, Identification of DNA adducts of acetaldehyde, Chemical research in toxicology, 13 (2000) 1149-1157.
[120] S.S. Hecht, E.J. McIntee, M. Wang, New DNA adducts of crotonaldehyde and acetaldehyde, Toxicology, 166 (2001) 31-36.
[121] P.J. Brooks, DNA Damage, DNA Repair, and Alcohol Toxicity—A Review, Alcoholism: Clinical and Experimental Research, 21 (1997) 1073-1082.
[122] M.S. Cooke, M.D. Evans, M. Dizdaroglu, J. Lunec, Oxidative DNA damage: mechanisms, mutation, and disease, The FASEB Journal, 17 (2003) 1195-1214.
[123] P.C. Dedon, S.R. Tannenbaum, Reactive nitrogen species in the chemical biology of inflammation, Archives of Biochemistry and Biophysics, 423 (2004) 12-22.
[124] M. Masuda, H. Nishino, H. Ohshima, Formation of 8-nitroguanosine in cellular RNA as a biomarker of exposure to reactive nitrogen species, Chemico-Biological Interactions, 139 (2002) 187-197.
[125] U. Nair, H. Bartsch, J. Nair, Lipid peroxidation-induced DNA damage in cancer-prone inflammatory diseases: A review of published adduct types and levels in humans, Free Radical Biology and Medicine, 43 (2007) 1109-1120.
[126] L.J. Marnett, Lipid peroxidation-DNA damage by malondialdehyde, Mutation research, 424 (1999) 83-95.
[127] L.J. Marnett, Oxy radicals, lipid peroxidation and DNA damage, Toxicology, 181-182 (2002) 219-222.
[128] T.B. Kryston, A.B. Georgiev, P. Pissis, A.G. Georgakilas, Role of oxidative stress and DNA damage in human carcinogenesis, Mutation research, 711 (2011) 193-201.
[129] H. Bartsch, J. Nair, Oxidative stress and lipid peroxidation-derived DNA-lesions in inflammation driven carcinogenesis, Cancer Detection and Prevention, 28 (2004) 385-391.
[130] D.E. Corpet, Red meat and colon cancer: should we become vegetarians, or can we make meat safer?, Meat science, 89 (2011) 310-316.
[131] D. Mandair, R.E. Rossi, M. Pericleous, T. Whyand, M.E. Caplin, Prostate cancer and the influence of dietary factors and supplements: a systematic review, Nutrition & metabolism, 11 (2014) 30.
[132] A. Tappel, Heme of consumed red meat can act as a catalyst of oxidative damage and could initiate colon, breast and prostate cancers, heart disease and other diseases, Medical hypotheses, 68 (2007) 562-564.
[133] R. Micha, S.K. Wallace, D. Mozaffarian, Red and processed meat consumption and risk of incident coronary heart disease, stroke, and diabetes mellitus: a systematic review and meta-analysis, Circulation, 121 (2010) 2271-2283.
[134] S.C. Larsson, N. Orsini, A. Wolk, Processed Meat Consumption and Stomach Cancer Risk: A Meta-Analysis, Journal of the National Cancer Institute, 98 (2006) 1078-1087.
[135] S.C. Larsson, A. Wolk, Red and processed meat consumption and risk of pancreatic cancer: meta-analysis of prospective studies, British journal of cancer, 106 (2012) 603-607.
[136] F. Levi, C. Pasche, C. La Vecchia, F. Lucchini, S. Franceschi, P. Monnier, Food groups and risk of oral and pharyngeal cancer, International Journal of Cancer, 77 (1998) 705-709.

[137] T. Van Hecke, E. Vossen, J. Vanden Bussche, K. Raes, L. Vanhaecke, S. De Smet, Fat content and nitrite-curing influence the formation of oxidation products and NOC-specific DNA adducts during in vitro digestion of meat, PloS one, 9 (2014) e101122.
[138] Z. Djuric, J.B. Depper, V. Uhley, D. Smith, S. Lababidi, S. Martino, L.K. Heilbrun, Oxidative DNA Damage Levels in Blood from Women at High Risk for Breast Cancer are Associated with Dietary intakes of Meats, Vegetables, and Fruits, Journal of the American Dietetic Association, 98 (1998) 524-528.
[139] A.M. Jenkinson, A.R. Collins, S.J. Duthie, K.W. Wahle, G.G. Duthie, The effect of increased intakes of polyunsaturated fatty acids and vitamin E on DNA damage in human lymphocytes, FASEB journal : official publication of the Federation of American Societies for Experimental Biology, 13 (1999) 2138-2142.
[140] S. Loft, E.B. Thorling, H.E. Poulsen, High fat diet induced oxidative DNA damage estimated by 8-oxo-7,8-dihydro-2'-deoxyguanosine excretion in rats, Free Radical Research, 29 (1998) 595-600.
[141] J. Vanden Bussche, L.Y. Hemeryck, T. Van Hecke, G.G. Kuhnle, F. Pasmans, S.A. Moore, T. Van de Wiele, S. De Smet, L. Vanhaecke, O(6)-carboxymethylguanine DNA adduct formation and lipid peroxidation upon in vitro gastrointestinal digestion of haem-rich meat, Molecular nutrition & food research, 58 (2014) 1883-1896.
[142] F. Drabløs, E. Feyzi, P.A. Aas, C.B. Vaagbø, B. Kavli, M.S. Bratlie, J. Peña-Diaz, M. Otterlei, G. Slupphaug, H.E. Krokan, Alkylation damage in DNA and RNA—repair mechanisms and medical significance, DNA Repair, 3 (2004) 1389-1407.
[143] P.B. Farmer, DNA and protein adducts as markers of genotoxicity, Toxicology letters, 149 (2004) 3-9.
[144] M.H. Lewin, N. Bailey, T. Bandaletova, R. Bowman, A.J. Cross, J. Pollock, D.E.G. Shuker, S.A. Bingham, Red Meat Enhances the Colonic Formation of the DNA Adduct O6-Carboxymethyl Guanine: Implications for Colorectal Cancer Risk, Cancer Research, 66 (2006) 1859-1865.
[145] G.P. Margison, M.F. Santibanez Koref, A.C. Povey, Mechanisms of carcinogenicity/chemotherapy by O6-methylguanine, Mutagenesis, 17 (2002) 483-487.
[146] F. Ender, G. Havre, A. Helgebostad, N. Koppang, R. Madsen, L. Ceh, Isolation and identification of a hepatotoxic factor in herring meal produced from sodium nitrite preserved herring, Naturwissenschaften, 51 (1964) 637-638.
[147] W. Lijinsky, Chemistry and biology of N-nitroso compounds, Cambridge Monographs on Cancer Research, (1992) 480.
[148] P.N. Magee, J.M. Barnes, The production of malignant primary hepatic tumours in the rat by feeding dimethylnitrosamine, British journal of cancer, 10 (1956) 114-122.
[149] W. Lijinsky, N-Nitroso compounds in the diet, Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 443 (1999) 129-138.
[150] J.H. Hotchkiss, Preformed N-nitroso compounds in foods and beverages, Cancer surveys, 8 (1989) 295-321.
[151] IARC, IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Humans: Some N-nitroso Compounds, IARC Scientific Publications, 17 (1978).
[152] P. Louis, G.L. Hold, H.J. Flint, The gut microbiota, bacterial metabolites and colorectal cancer, Nat Rev Micro, 12 (2014) 661-672.
[153] R. Goldman, P.G. Shields, Food mutagens, The Journal of nutrition, 133 Suppl 3 (2003) 965s-973s.
[154] M. Valko, M. Izakovic, M. Mazur, C. Rhodes, J. Telser, Role of oxygen radicals in DNA damage and cancer incidence, Mol Cell Biochem, 266 (2004) 37-56.
[155] L.J. Marnett, Oxyradicals and DNA damage, Carcinogenesis, 21 (2000) 361-370.

[156] F.L. Chung, J. Pan, S. Choudhury, R. Roy, W. Hu, M.S. Tang, Formation of trans-4-hydroxy-2-nonenal- and other enal-derived cyclic DNA adducts from omega-3 and omega-6 polyunsaturated fatty acids and their roles in DNA repair and human p53 gene mutation, Mutation research, 531 (2003) 25-36.
[157] D. Pluskota-Karwatka, A.J. Pawlowicz, L. Kronberg, Formation of malonaldehyde-acetaldehyde conjugate adducts in calf thymus DNA, Chemical research in toxicology, 19 (2006) 921-926.
[158] A.S. Jones, P. Lewis, S.F. Withers, The synthesis of carboxymethyl derivatives of purines and pyrimidines and their condensation with naturally occurring macromolecules, Tetrahedron, 29 (1973) 2293-2296.
[159] C. Zhao, M. Tyndyk, I. Eide, K. Hemminki, Endogenous and background DNA adducts by methylating and 2-hydroxyethylating agents, Mutation research, 424 (1999) 117-125.
[160] P.J. Brooks, J.A. Theruvathu, DNA adducts from acetaldehyde: implications for alcohol-related carcinogenesis, Alcohol, 35 (2005) 187-193.
[161] P.J. Aduma, S.V. Gupta, A.L. Stuart, G. Tourigny, Regulatory effects of deoxyribonucleosides on the activity of 5-methoxymethyl-2'-deoxycytidine: modulation of antiherpes activity by deoxyguanosine and tetrahydrodeoxyuridine, Antiviral research, 15 (1991) 301-313.
[162] L. Airoldi, C. Magagnotti, M. Bonfanti, L. Chiappetta, M. Lolli, C. Medana, G. De Gregorio, R. Fanelli, Detection of O6-butyl- and O6-(4-hydroxybutyl)guanine in urothelial and hepatic DNA of rats given the bladder carcinogen N-nitrosobutyl(4-hydroxybutyl)amine, Carcinogenesis, 15 (1994) 2297-2301.
[163] A. Kopplin, G. Eberle-Adamkiewicz, K.-H. Glüsenkamp, P. Nehls, U. Kirstein, Urinary excretion of 3-methyladenine and 3-ethyladenine after controlled exposure to tobacco smoke, Carcinogenesis, 16 (1995) 2637-2641.
[164] N.B. Perry, J.W. Blunt, M.H.G. Munro, 1,3,7-Trimethylguanine from the Sponge Latrunculia brevis, Journal of Natural Products, 50 (1987) 307-308.
[165] B. Lesyng, W. Saenger, Theoretical investigations on the conformation of 1,5,N(4),N(4)-tetramethylcytosine, Biochimica et biophysica acta, 544 (1978) 215-224.
[166] A. Segal, J.J. Solomon, U. Maté, Isolation of 3-(2-carboxyethyl)thymine following in vitro reaction of β-propiolactone with calf thymus DNA, Chemico-Biological Interactions, 29 (1980) 335-346.
[167] D. Palli, C. Saieva, C. Coppi, G. Del Giudice, C. Magagnotti, G. Nesi, F. Orsi, L. Airoldi, O6-alkylguanines, dietary N-nitroso compounds, and their precursors in gastric cancer, Nutrition and cancer, 39 (2001) 42-49.
[168] H.A. Billson, K.L. Harrison, N.P. Lees, C.N. Hall, G.P. Margison, A.C. Povey, Dietary variables associated with DNA N7-methylguanine levels and O6-alkylguanine DNA-alkyltransferase activity in human colorectal mucosa, Carcinogenesis, 30 (2009) 615-620.
[169] W.Y. Hsu, W.T. Chen, W.D. Lin, F.J. Tsai, Y. Tsai, C.T. Lin, W.Y. Lo, L.B. Jeng, C.C. Lai, Analysis of urinary nucleosides as potential tumor markers in human colorectal cancer by high performance liquid chromatography/electrospray ionization tandem mass spectrometry, Clinica chimica acta; international journal of clinical chemistry, 402 (2009) 31-37.
[170] G. Cheng, M. Wang, P.W. Villalta, S.S. Hecht, Detection of 7-(2'-Carboxyethyl)guanine but Not 7-Carboxymethylguanine in Human Liver DNA, Chemical research in toxicology, 23 (2010) 1089-1096.
[171] B. Spilsberg, T. Rundberget, L.E. Johannessen, A.B. Kristoffersen, A. Holst-Jensen, K.G. Berdal, Detection of Food-Derived Damaged Nucleosides with Possible Adverse Effects on Human Health Using a Global Adductomics Approach, Journal of Agricultural and Food Chemistry, 58 (2010) 6370-6375.

[172] K. Arashidani, N. Iwamoto-Tanaka, M. Muraoka, H. Kasai, Genotoxicity of ribo- and deoxyribonucleosides of 8-hydroxyguanine, 5-hydroxycytosine, and 2-hydroxyadenine: induction of SCE in human lympgocytes and mutagenicity in Salmonella typhimurium TA 100, Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 403 (1998) 223-227.
[173] M. Inoue, H. Kamiya, K. Fujikawa, Y. Ootsuyama, N. Murata-Kamiya, T. Osaki, K. Yasumoto, H. Kasai, Induction of chromosomal gene mutations in Escherichia coli by direct incorporation of oxidatively damaged nucleotides. New evaluation method for mutagenesis by damaged DNA precursors in vivo, The Journal of biological chemistry, 273 (1998) 11069-11074.
[174] A. Klungland, R. Paulsen, V. Rolseth, Y. Yamada, Y. Ueno, P. Wiik, A. Matsuda, E. Seeberg, S. Bjelland, 5-Formyluracil and its nucleoside derivatives confer toxicity and mutagenicity to mammalian cells by interfering with normal RNA and DNA metabolism, Toxicology letters, 119 (2001) 71-78.
[175] C. Heidelberger, Cancer Chemotherapy with Purine and Pyrimidine Analogues, Annual Review of Pharmacology, 7 (1967) 101-124.
[176] C. Périgaud, G. Gosselin, J.L. Imbach, Nucleoside Analogues as Chemotherapeutic Agents: A Review, Nucleosides and Nucleotides, 11 (1992) 903-945.
[177] B. Tudek, E. Speina, Oxidatively damaged DNA and its repair in colon carcinogenesis, Mutation research, 736 (2012) 82-92.
[178] D.F. Merlo, S. Agramunt, L. Anna, H. Besselink, M. Botsivali, N.J. Brady, M. Ceppi, L. Chatzi, B. Chen, I. Decordier, P.B. Farmer, S. Fleming, V. Fontana, A. Forsti, E. Fthenou, F. Gallo, P. Georgiadis, H. Gmuender, R.W. Godschalk, B. Granum, L.J. Hardie, K. Hemminki, K. Hochstenbach, L.E. Knudsen, M. Kogevinas, K. Kovacs, S.A. Kyrtopoulos, M. Lovik, J.K. Nielsen, U.C. Nygaard, M. Pedersen, P. Rydberg, B. Schoket, D. Segerback, R. Singh, J. Sunyer, M. Tornqvist, H. van Loveren, F.J. van Schooten, K. Vande Loock, H. von Stedingk, J. Wright, J.C. Kleinjans, M. Kirsch-Volders, J.H. van Delft, Micronuclei in cord blood lymphocytes and associations with biomarkers of exposure to carcinogens and hormonally active factors, gene polymorphisms, and gene expression: the NewGeneris cohort, Environmental health perspectives, 122 (2014) 193-200.
[179] V. Ho, S. Peacock, T.E. Massey, J.E. Ashbury, S.J. Vanner, W.D. King, Meat-derived carcinogens, genetic susceptibility and colorectal adenoma risk, Genes & nutrition, 9 (2014) 430.
[180] S. Balbo, P. Brooks, Implications of Acetaldehyde-Derived DNA Adducts for Understanding Alcohol-Related Carcinogenesis, in: V. Vasiliou, S. Zakhari, H.K. Seitz, J.B. Hoek (Eds.) Biological Basis of Alcohol-Induced Cancer, Springer International Publishing2015, pp. 71-88.
[181] H. Seitz, S. Mueller, Alcohol and Cancer: An Overview with Special Emphasis on the Role of Acetaldehyde and Cytochrome P450 2E1, in: V. Vasiliou, S. Zakhari, H.K. Seitz, J.B. Hoek (Eds.) Biological Basis of Alcohol-Induced Cancer, Springer International Publishing2015, pp. 59-70.
[182] G.M. Williams, M.J. Iatropoulos, A.M. Jeffrey, J.D. Duan, Methyleugenol hepatocellular cancer initiating effects in rat liver, Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association, 53 (2013) 187-196.
[183] H.H. Schmeiser, J.E. Kucab, V.M. Arlt, D.H. Phillips, M. Hollstein, G. Gluhovschi, C. Gluhovschi, M. Modilca, L. Daminescu, L. Petrica, S. Velciov, Evidence of exposure to aristolochic acid in patients with urothelial cancer from a Balkan endemic nephropathy region of Romania, Environmental and molecular mutagenesis, 53 (2012) 636-641.
[184] T.J. Key, A. Schatzkin, W.C. Willett, N.E. Allen, E.A. Spencer, R.C. Travis, Diet, nutrition and the prevention of cancer, Public Health Nutr, 7 (2004) 187-200.
[185] W.C. Willett, Diet and cancer, The oncologist, 5 (2000) 393-404.

[186] W.C. Willett, Balancing life-style and genomics research for disease prevention, Science (New York, N.Y.), 296 (2002) 695-698.
[187] Food, Nutrition, Physical Activity, and the Prevention of Cancer: A Global Perspective World Cancer Research Fund/American Institute for Cancer Research, Washington DC, 2007.
[188] A.C. Society, Colorectal Cancer Facts & Figures 2014-2016.
[189] IARC, IARC Monographs evaluate consumption of red meat and processed meat, Lyon, 2015.
[190] N.M. Bastide, F.H. Pierre, D.E. Corpet, Heme iron from meat and risk of colorectal cancer: a meta-analysis and a review of the mechanisms involved, Cancer prevention research (Philadelphia, Pa.), 4 (2011) 177-184.
[191] D. Demeyer, B. Mertens, S. De Smet, M. Ulens, Mechanisms Linking Colorectal Cancer to the Consumption of (Processed) Red Meat: A Review, Critical reviews in food science and nutrition, 56 (2016) 2747-2766.
[192] L.R. Ferguson, Meat and cancer, Meat science, 84 (2010) 308-313.
[193] D.D. Alexander, C.A. Cushing, K.A. Lowe, B. Sceurman, M.A. Roberts, Meta-analysis of animal fat or animal protein intake and colorectal cancer, The American journal of clinical nutrition, 89 (2009) 1402-1409.
[194] K. Windey, V. De Preter, K. Verbeke, Relevance of protein fermentation to gut health, Molecular nutrition & food research, 56 (2012) 184-196.
[195] G.G. Kuhnle, S.A. Bingham, Dietary meat, endogenous nitrosation and colorectal cancer, Biochemical Society transactions, 35 (2007) 1355-1357.
[196] N.M. Bastide, F. Chenni, M. Audebert, R.L. Santarelli, S. Tache, N. Naud, M. Baradat, I. Jouanin, R. Surya, D.A. Hobbs, G.G. Kuhnle, I. Raymond-Letron, F. Gueraud, D.E. Corpet, F.H. Pierre, A central role for heme iron in colon carcinogenesis associated with red meat intake, Cancer Res, 75 (2015) 870-879.
[197] F. Pierre, A. Freeman, S. Tache, R. Van der Meer, D.E. Corpet, Beef meat and blood sausage promote the formation of azoxymethane-induced mucin-depleted foci and aberrant crypt foci in rat colons, The Journal of nutrition, 134 (2004) 2711-2716.
[198] F. Pierre, G. Peiro, S. Tache, A.J. Cross, S.A. Bingham, N. Gasc, G. Gottardi, D.E. Corpet, F. Gueraud, New marker of colon cancer risk associated with heme intake: 1,4-dihydroxynonane mercapturic acid, Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology, 15 (2006) 2274-2279.
[199] J. Kanner, Oxidative processes in meat and meat products: Quality implications, Meat science, 36 (1994) 169-189.
[200] L.J. Marnett, Inflammation and Cancer: Chemical Approaches to Mechanisms, Imaging, and Treatment, The Journal of Organic Chemistry, 77 (2012) 5224-5238.
[201] A.J. Cross, J.R. Pollock, S.A. Bingham, Haem, not protein or inorganic iron, is responsible for endogenous intestinal N-nitrosation arising from red meat, Cancer Res, 63 (2003) 2358-2360.
[202] A.M. Joosen, G.G. Kuhnle, S.M. Aspinall, T.M. Barrow, E. Lecommandeur, A. Azqueta, A.R. Collins, S.A. Bingham, Effect of processed and red meat on endogenous nitrosation and DNA damage, Carcinogenesis, 30 (2009) 1402-1407.
[203] S.A. Bingham, R. Hughes, A.J. Cross, Effect of White Versus Red Meat on Endogenous N-Nitrosation in the Human Colon and Further Evidence of a Dose Response, The Journal of nutrition, 132 (2002) 3522S-3525S.
[204] R. Hughes, A.J. Cross, J.R. Pollock, S. Bingham, Dose-dependent effect of dietary meat on endogenous colonic N-nitrosation, Carcinogenesis, 22 (2001) 199-202.
[205] R.L. Santarelli, J.-L. Vendeuvre, N. Naud, S. Taché, F. Guéraud, M. Viau, C. Genot, D.E. Corpet, F.H.F. Pierre, Meat processing and colon carcinogenesis: cooked, nitrite-treated, and

oxidized high-heme cured meat promotes mucin-depleted foci in rats, Cancer prevention research (Philadelphia, Pa.), 3 (2010) 852-864.
[206] F.H. Pierre, R.L. Santarelli, O. Allam, S. Tache, N. Naud, F. Gueraud, D.E. Corpet, Freeze-dried ham promotes azoxymethane-induced mucin-depleted foci and aberrant crypt foci in rat colon, Nutrition and cancer, 62 (2010) 567-573.
[207] E. Byres, A.W. Paton, J.C. Paton, J.C. Lofling, D.F. Smith, M.C. Wilce, U.M. Talbot, D.C. Chong, H. Yu, S. Huang, X. Chen, N.M. Varki, A. Varki, J. Rossjohn, T. Beddoe, Incorporation of a non-human glycan mediates human susceptibility to a bacterial toxin, Nature, 456 (2008) 648-652.
[208] M. Hedlund, V. Padler-Karavani, N.M. Varki, A. Varki, Evidence for a human-specific mechanism for diet and antibody-mediated inflammation in carcinoma progression, Proc Natl Acad Sci U S A, 105 (2008) 18936-18941.
[209] K. Puangsombat, P. Gadgil, T.A. Houser, M.C. Hunt, J.S. Smith, Occurrence of heterocyclic amines in cooked meat products, Meat science, 90 (2012) 739-746.
[210] S.M. Tabatabaei, J.S. Heyworth, M.W. Knuiman, L. Fritschi, Dietary Benzo[a]pyrene Intake from Meat and the Risk of Colorectal Cancer, Cancer Epidemiology Biomarkers & Prevention, 19 (2010) 3182-3184.
[211] T. Kumagai, Y. Kawamoto, Y. Nakamura, I. Hatayama, K. Satoh, T. Osawa, K. Uchida, 4-Hydroxy-2-nonenal, the End Product of Lipid Peroxidation, Is a Specific Inducer of Cyclooxygenase-2 Gene Expression, Biochemical and biophysical research communications, 273 (2000) 437-441.
[212] F.W. Wiese, P.A. Thompson, F.F. Kadlubar, Carcinogen substrate specificity of human COX-1 and COX-2, Carcinogenesis, 22 (2001) 5-10.
[213] A. Ferrandez, S. Prescott, R.W. Burt, COX-2 and colorectal cancer, Curr Pharm Des, 9 (2003) 2229-2251.
[214] S. Balbo, R.J. Turesky, P.W. Villalta, DNA adductomics, Chemical research in toxicology, 27 (2014) 356-366.
[215] H. Koc, J.A. Swenberg, Applications of mass spectrometry for quantitation of DNA adducts, Journal of Chromatography B, 778 (2002) 323-343.
[216] M.W. Himmelstein, P.J. Boogaard, J. Cadet, P.B. Farmer, J.H. Kim, E.A. Martin, R. Persaud, D.E. Shuker, Creating context for the use of DNA adduct data in cancer risk assessment: II. Overview of methods of identification and quantitation of DNA damage, Critical reviews in toxicology, 39 (2009) 679-694.
[217] P.B. Farmer, R. Singh, Use of DNA adducts to identify human health risk from exposure to hazardous environmental pollutants: The increasing role of mass spectrometry in assessing biologically effective doses of genotoxic carcinogens, Mutation Research/Reviews in Mutation Research, 659 (2008) 68-76.
[218] R. Singh, P.B. Farmer, Liquid chromatography-electrospray ionization-mass spectrometry: the future of DNA adduct detection, Carcinogenesis, 27 (2006) 178-196.
[219] R. Singh, F. Teichert, A. Seidel, J. Roach, R. Cordell, M.K. Cheng, H. Frank, W.P. Steward, M.M. Manson, P.B. Farmer, Development of a targeted adductomic method for the determination of polycyclic aromatic hydrocarbon DNA adducts using online column-switching liquid chromatography/tandem mass spectrometry, Rapid communications in mass spectrometry : RCM, 24 (2010) 2329-2340.
[220] S. Balbo, S.S. Hecht, P. Upadhyaya, P.W. Villalta, Application of a High-Resolution Mass-Spectrometry-Based DNA Adductomics Approach for Identification of DNA Adducts in Complex Mixtures, Analytical Chemistry, 86 (2014) 1744-1752.
[221] E.E. Bessette, A.K. Goodenough, S. Langouet, I. Yasa, I.D. Kozekov, S.D. Spivack, R.J. Turesky, Screening for DNA adducts by data-dependent constant neutral loss-triple stage mass

spectrometry with a linear quadrupole ion trap mass spectrometer, Anal Chem, 81 (2009) 809-819.
[222] M. Goggin, R. Loeber, S. Park, V. Walker, J. Wickliffe, N. Tretyakova, HPLC-ESI+-MS/MS analysis of N7-guanine-N7-guanine DNA cross-links in tissues of mice exposed to 1,3-butadiene, Chemical research in toxicology, 20 (2007) 839-847.
[223] S. Inagaki, H. Hirashima, Y. Esaka, T. Higashi, J. Min, T. Toyo'oka, Screening DNA Adducts by LC–ESI–MS–MS: Application to Screening New Adducts Formed from Acrylamide, Chroma, 72 (2010) 1043-1048.
[224] P. Jaruga, G. Kirkali, M. Dizdaroglu, Measurement of formamidopyrimidines in DNA, Free radical biology & medicine, 45 (2008) 1601-1609.
[225] J. Wang, B. Yuan, C. Guerrero, R. Bahde, S. Gupta, Y. Wang, Quantification of oxidative DNA lesions in tissues of Long-Evans Cinnamon rats by capillary high-performance liquid chromatography-tandem mass spectrometry coupled with stable isotope-dilution method, Anal Chem, 83 (2011) 2201-2209.
[226] J.J. Klaene, V.K. Sharma, J. Glick, P. Vouros, The analysis of DNA adducts: the transition from (32)P-postlabeling to mass spectrometry, Cancer letters, 334 (2013) 10-19.
[227] V.K. Sharma, J. Glick, Q. Liao, C. Shen, P. Vouros, GenoMass software: a tool based on electrospray ionization tandem mass spectrometry for characterization and sequencing of oligonucleotide adducts, Journal of mass spectrometry : JMS, 47 (2012) 490-501.
[228] N. Tretyakova, P.W. Villalta, S. Kotapati, Mass Spectrometry of Structurally Modified DNA, Chemical Reviews, 113 (2013) 2395-2436.
[229] K.-Y. Wu, S.-Y. Chiang, W.-C. Shih, C.-C.J. Huang, M.-F. Chen, J.A. Swenberg, The application of mass spectrometry in molecular dosimetry: Ethylene oxide as an example, Mass Spectrometry Reviews, 30 (2011) 733-756.
[230] K. Dettmer, P.A. Aronov, B.D. Hammock, Mass spectrometry-based metabolomics, Mass Spectrom Rev, 26 (2007) 51-78.
[231] J. Vanden Bussche, S.A. Moore, F. Pasmans, G.G. Kuhnle, L. Vanhaecke, An approach based on ultra-high pressure liquid chromatography-tandem mass spectrometry to quantify O6-methyl and O6-carboxymethylguanine DNA adducts in intestinal cell lines, Journal of chromatography. A, 1257 (2012) 25-33.
[232] H. Mao, N.C. Schnetz-Boutaud, J.P. Weisenseel, L.J. Marnett, M.P. Stone, Duplex DNA catalyzes the chemical rearrangement of a malondialdehyde deoxyguanosine adduct, Proceedings of the National Academy of Sciences, 96 (1999) 6615-6620.
[233] L.J. Niedernhofer, M. Riley, N. Schnetz-Boutaud, G. Sanduwaran, A.K. Chaudhary, G.R. Reddy, L.J. Marnett, Temperature-dependent formation of a conjugate between tris(hydroxymethyl)aminomethane buffer and the malondialdehyde-DNA adduct pyrimidopurinone, Chemical research in toxicology, 10 (1997) 556-561.
[234] P.B. Inskeep, N. Koga, J.L. Cmarik, F.P. Guengerich, Covalent binding of 1,2-dihaloalkanes to DNA and stability of the major DNA adduct, S-[2-(N7-guanyl)ethyl]glutathione, Cancer Res, 46 (1986) 2839-2844.
[235] F. Bianchini, R. Montesano, D.E. Shuker, J. Cuzick, C.P. Wild, Quantification of 7-methyldeoxyguanosine using immunoaffinity purification and HPLC with electrochemical detection, Carcinogenesis, 14 (1993) 1677-1682.
[236] A. Izzotti, M. Bagnasco, L. Scatolini, A. Rovida, S. De Flora, Post-mortem stability of benzo[a]pyrene diolepoxide--DNA adducts in rat organs, Carcinogenesis, 14 (1993) 2185-2187.
[237] M.R. Osborne, D.H. Phillips, Preparation of a Methylated DNA Standard, and Its Stability on Storage, Chemical research in toxicology, 13 (2000) 257-261.

[238] P.R. Hillestrøm, A. Weimann, H.E. Poulsen, Quantification of Urinary Etheno-DNA Adducts by Column-Switching LC/APCI-MS/MS, Journal of the American Society for Mass Spectrometry, 17 (2006) 605-610.
[239] B. Binkova, F. Hubalek, R. Sram, Stability of benzo[a]pyrene DNA adducts in rat tissues during their long-term storage at -20 degrees C or -80 degrees C, Mutation research, 371 (1996) 229-235.
[240] F. Zhang, M.J. Bartels, L.H. Pottenger, B.B. Gollapudi, M.R. Schisler, Simultaneous quantitation of 7-methyl- and O6-methylguanine adducts in DNA by liquid chromatography-positive electrospray tandem mass spectrometry, Journal of chromatography. B, Analytical technologies in the biomedical and life sciences, 833 (2006) 141-148.
[241] K. Kato, E. Yamamura, M. Kawanishi, T. Yagi, T. Matsuda, A. Sugiyama, Y. Uno, Application of the DNA adductome approach to assess the DNA-damaging capability of in vitro micronucleus test-positive compounds, Mutation research, 721 (2011) 21-26.
[242] R. Singh, M. McEwan, J.H. Lamb, R.M. Santella, P.B. Farmer, An improved liquid chromatography/tandem mass spectrometry method for the determination of 8-oxo-7,8-dihydro-2'-deoxyguanosine in DNA samples using immunoaffinity column purification, Rapid Communications in Mass Spectrometry, 17 (2003) 126-134.
[243] L.A. Liotta, E.F. Petricoin, 3rd, -Omics and cancer biomarkers: link to the biological truth or bear the consequences, Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology, 21 (2012) 1229-1235.
[244] R.A. Kanaly, R. Micheletto, T. Matsuda, Y. Utsuno, Y. Ozeki, N. Hamamura, Application of DNA adductomics to soil bacterium Sphingobium sp. strain KK22, MicrobiologyOpen, 4 (2015) 841-856.
[245] J.C. Rockett, M.E. Burczynski, A.J. Fornace, P.C. Herrmann, S.A. Krawetz, D.J. Dix, Surrogate tissue analysis: monitoring toxicant exposure and health status of inaccessible tissues through the analysis of accessible tissues and cells, Toxicology and applied pharmacology, 194 (2004) 189-199.
[246] J.K. Wiencke, K.T. Kelsey, A. Varkonyi, K. Semey, J.C. Wain, E. Mark, D.C. Christiani, Correlation of DNA adducts in blood mononuclear cells with tobacco carcinogen-induced damage in human lung, Cancer Res, 55 (1995) 4910-4914.
[247] F.J. van Schooten, M.J. Hillebrand, F.E. van Leeuwen, N. van Zandwijk, H.M. Jansen, L. den Engelse, E. Kriek, Polycyclic aromatic hydrocarbon--DNA adducts in white blood cells from lung cancer patients: no correlation with adduct levels in lung, Carcinogenesis, 13 (1992) 987-993.
[248] US Food and Drug Administration, Draft Guidance for Industry for Bioanalytical Method Validation, www.fda.gov (2013).
[249] E.M.A. Committee for Medicinal Products for Human Use, Concept paper on good genomic biomarker practices, www.ema.europe.eu, (2014).

CHAPTER II

Cytotoxic, genotoxic and metabolic effects upon digestion of heme-rich meat

Adapted from:

Vanden Bussche J*, **Hemeryck LY***, Van Hecke T, Kuhnle GG, Pasmans F, Moore SA, Van de Wiele T, De Smet S, Vanhaecke L. O^6-carboxymethylguanine DNA adduct formation and lipid peroxidation upon in vitro gastrointestinal digestion of haem-rich meat. Mol Nutr Food Res. 2014 Sep;58(9):1883-96. *Shared first author.

ABSTRACT

Epidemiological and clinical studies have demonstrated that the consumption of heme-rich meat may contribute to the risk of colorectal cancer. Two hypotheses have been put forward to explain this causal relationship, i.e. NOC formation and lipid peroxidation. In this study, the NOC-related DNA adduct O^6-CMG and the LPO product MDA were measured in individual *in vitro* gastrointestinal digestions of meat types varying in heme content (beef, pork, chicken). While MDA formation peaked during the *in vitro* small intestinal digestion, alkylation and concomitant DNA adduct formation was observed in seven (out of 15) individual colonic digestions using separate fecal inocula. From those, two heme-rich meat digestions demonstrated a significantly higher O^6-CMG formation ($p < 0.05$). MDA concentrations proved to be positively correlated ($p < 0.0004$) with heme content of digested meat. The addition of myoglobin, a heme-containing protein, to the digestive simulation showed a dose–response association with O^6-CMG ($p = 0.004$) and MDA ($p = 0.008$) formation. The results suggest the heme-iron involvement for both the LPO and NOC pathway during meat digestion. Moreover, results unambiguously demonstrate that DNA adduct formation is very prone to inter-individual variation, suggesting a person-dependent susceptibility to colorectal cancer development following heme-rich meat consumption.

Keywords:

In vitro gastrointestinal digestion, Malondialdehyde, Meat consumption, N-nitroso compounds, O^6-carboxymethylguanine

1. INTRODUCTION

Epidemiological and clinical studies have demonstrated that the consumption of meat, and in particular red and processed meat is associated with certain health risks [1, 2]. Regular or high consumption of red and processed meat has been linked to the risk of CRC [1], coronary heart disease and also type 2 diabetes [2-4]. The link between red meat consumption and CRC on the one hand and red meat and type 2 diabetes on the other might even be intertwined [5, 6]. As a plausible origin for the increased CRC risk, the formation of endogenous NOCs has been hypothesized, since a dose–response relationship with the fecal excretion of NOCs for red and processed meat but not for white meat intake has been established [7-9]. The role of heme, abundantly present in red beef meat (1.8 mg/100 g) [10], in the fecal excretion of NOCs has been confirmed [11]. Heme can become readily nitrosylated and act as a nitrosating agent and thus promote the formation of NOCs [12]. A high-heme diet has also been suggested as a mechanistic basis in the observed correlation between lipid peroxidation (LPO) and increased CRC risk [13].

NOCs, such as nitrosamines, nitrosamides or nitrosoguanidines, are known to be potent carcinogens. The alkylation of DNA is likely to be a major effect of metabolized N-alkyl-NOCs, which are able to interact with the nucleophilic centres of DNA bases [14, 15]. In addition, several nitrosated glycine derivatives have been shown to react with DNA *in vitro* to give NOC-derived, i.e. carboxymethyl (e.g. O^6-carboxymethyl-2'-deoxyguanosine, O^6-CMdG), and to a lesser extent methyl adducts (e.g. O^6-methyl-2'-deoxyguanosine, O^6-MedG) [15-17]. Since glycine is one of the most common dietary amino acids, it would appear likely that nitrosation products of glycine would constitute a major source of alkylating agents in the human gastrointestinal tract (GIT) [18, 19]. This was reinforced by the detection of O^6-CMdG and O^6-methyl-2'-deoxyguanosine in colonic biopsies and human blood DNA [17, 20, 21]. Additionally, O^6-CMdG is not repaired by either bacterial or mammalian O^6-methylguanine-DNA methyltransferase (MGMT) under *in vitro* conditions suggesting that this adduct is likely to accumulate in cellular DNA of GIT tissue and possibly represents a promutagenic lesion [15, 21]. The identification and quantification of very low DNA adduct levels, *in vivo* or *in vitro* requires ultrasensitive methodologies. This is in particular true for the analysis of human samples or *in vitro* applications on cell lines, where only small amounts of sample, and therefore DNA, are available. The different methods currently used for DNA adduct analysis include, i.e. immunoassays [22, 23], ^{32}P-postlabelling [24], GC-ECD [25] and HPLC with fluorescence detection [16, 26]. For several

years now, mass spectrometric detection has been playing an increasingly important role in the field of DNA adduct detection [27-30], since it achieves a perfect balance between a high specificity and sensitivity. Because of this, we developed an UHPLC-MS/MS method for the simultaneous detection of O^6-methyl-G (O^6-MeG) and O^6-CMG adducts, with LODs of 30 and 50 fmol/mg DNA, respectively [31].

Lipids are biological targets of various reactive oxygen and nitrogen species. This oxidative stress leads to free radical chain reactions and subsequent formation of a vast array of by-products including aldehydes [32, 33]. The main aldehyde formed from the peroxidation of polyunsaturated fatty acids is MDA, a DNA-reactive product capable of forming exocyclic DNA adducts, of which most of these are anticipated to be highly mutagenic [34]. The mutagenicity and carcinogenicity of MDA has been confirmed in mammalian cells as well as in animals [34], and has been put forward as a plausible hypothesis for the link between high red meat consumption and CRC [13]. A variety of methods described in literature allow the detection and quantification of MDA in biological matrices. Nowadays, the most commonly employed method utilizes the reaction of MDA with thiobarbituric acid, resulting in the well-known thiobarbituric acid reactive substances (TBARS) assay [35]. Its product can be detected by colorimetry (532–535 nm) or fluorimetry (excitation at 532 nm and emission at 553 nm) [38]. It is an easy and inexpensive method, however MDA may be bound to matrix molecules and therefore undetectable without an adequate step to liberate it, such as alkaline hydrolysis [36].

Mechanistic studies investigating the correlation between red meat consumption and LPO as well as NOC-related DNA adduct formation are scarce. Therefore, during this study, i*n vitro* gastrointestinal digestive simulations of different meat types were undertaken to determine which specific digestive processes are involved in the formation of the harmful NOC-related DNA adducts O^6-MeG and O^6-CMG and the LPO product MDA. DNA adduct formation was assessed by quantitatively measuring O^6-MeG and O^6-CMG using LC-QqQ-MS/MS. The LPO product MDA was directly measured by means of a TBARS assay.

2. MATERIALS AND METHODS

2.1 Reagents and chemicals

(Caution: Potassium diazoacetate (KDA) is carcinogenic. It should be handled in a well-ventilated fume hood with extreme care and personal protective equipment.)

The chemical standard O^6-MeG was purchased from Sigma-Aldrich (St. Louis, MO, USA) and the internal standard O^6-methyl-d_3-guanine was obtained from Toronto Research Chemicals Inc. (Toronto, Canada). O^6-CMG standard was derived *via* acidic hydrolysis with 0.1 M formic acid at 70°C for 1 h, of O^6-carboxymethyl-2-deoxyguanosine (O^6-CMdG, purity > 96 %) [24]. The stock solutions of the chemical standards O^6-MeG and O^6-CMG were prepared in ethanol at a concentration of 6.06 and 4.8 mol/mL, respectively, and diluted with deionized water for obtaining working solutions of 818/81.8/8.18 fM and 646/64.6/6.46 fM, respectively. A working solution of O^6-methyl-d_3-guanine (118.9 fM) in deionized water was prepared as internal standard (IS). All solutions were stored in dark glass bottles at -20°C.

KDA, a known nitrosated glycine derivative [15, 17], was synthesized *via* alkaline hydrolysis of ethyldiazoacetate (EtDA) Sigma-Aldrich) [16]. The stock solution of 800 mM KDA was made up of 1.14 g of EtDA and 11.4 mL of 1.8 M KOH, and mixed for 4 h at room temperature in the dark. Working solutions were obtained through dilution with PBS buffer [16].

Reagents were of analytical grade (VWR International, Merck, Darmstadt, Germany) when used for extraction and purification, and of Optima R MS grade for MS application (Fisher Scientific UK, Loughborough, UK), respectively.

2.2 Incubation conditions

2.2.1 Collection and preparation of human fecal samples

Fecal samples were obtained from 15 healthy volunteers (ten males and five females) between the ages of 20 and 60. Donors were on a Western-type diet and none had a history of digestive pathology nor had received antibiotics during 6 months prior to the sampling. All donors of fecal material were recruited among the laboratory personnel and their family members through informal announcement, after which all participating volunteers gave their written informed consent. The obtained data and volunteer information were analyzed anonymously and de-

identified. The research was approved by the Federal Public Service of Health, Food Chain Safety and Environment, Belgium, but there was no need to submit an application to the ethical committee due to the non-invasive nature of the voluntary donation of fecal samples.

Fecal slurries of 20 % w/v fresh inocula were prepared by homogenizing (stomacher 400 Classic Laboratory Blender, Seward, West Sussex, UK) the feces with PBS (0.1 M, pH 7), containing 1 g/L sodium thioglycolate as reducing agent. The particulate matter and biomass were removed by centrifugation for 2 min at 500 x g. For storage purposes at -80°C, 20 % (v:v) of glycerol (99.5 %; Analar Normapur, Fontenaysous-Bois, France) was added to the supernatant as a cryoprotectant.

2.2.2 Simulated gastrointestinal digestion

Static *in vitro* incubation experiments of meat varying in heme content (chicken, beef and to a small extent pork) were performed in autoclaved penicillin flasks. The meat was therefore subjected to a sequential simulation of stomach, small and large intestinal digestion according to Van de Wiele et al. [37]. The different meat samples were obtained at a local butcher shop and their heme content was measured according to Hornsey [38]. A typical *in vitro* stomach digestion consisted of an incubation of 4 g of prepared meat (cooked for 10 min at 80°C to mimic a Western meal preparation) with pepsin (10 mg/L) for 2 h at pH 1.5 at a 1:10 ratio (v:v). Next, bile salts (6.0 g/L) and pancreatic enzymes supplemented as pancreatic powder of bovine origin (0.9 g/L) were added to the stomach suspension at a 1:2 ratio (v:v) to simulate small intestinal conditions. Small intestinal digestive simulation consisted of an incubation for 4 h at pH 7. Finally, for the colonic digestion, fecal microbiota (1:3 ratio (v:v)) and Simulator of the Human Intestinal Microbial Ecosystem (SHIME) broth (1:4 ratio (v:v)) were added to the small intestinal incubation mixture and incubation was continued for another 72 h after 1 h of N_2 flushing. The colon suspension contained *in vitro* cultured microbiota that was isolated from human feces and is considered representative of the *in vivo* colon microbial ecology after a growth stabilization period in Brain Heart Infusion (BHI) broth at a 1:9 ratio (v:v) in N_2 atmosphere. As a control, meat samples were incubated with inactive fecal microbiota. To this extent, fecal microbiota was autoclaved for 30 min (121°C, 1 bar overpressure). For investigating the heme-iron hypothesis, an additional *in vitro* digestion was performed with the fecal inoculum of an O^6-CMG-producing volunteer (ID n° 7, as listed in table 1). Besides the beef, different amounts of myoglobin were

supplemented at the start of the digestion, i.e. 0, 2.8, 14.1 and 28.3 nmol of myoglobin per mL of digestive fluid, respectively.

To avoid photocatalytic effects, all digestions were performed in amber flasks. Each batch culture was sealed with butylrubber stops and anaerobiosis was obtained by flushing the flasks with N_2 alternating every 2 min with vacuum suction during 1 h. Resazurin (2 mg/L) was added as redox indicator to the fecal slurry and to the colonic stage of the digestion. A pink colour indicated a redox potential higher than -80 mV, a colourless solution showed a redox potential below this limit, i.e. anaerobic. The redox potential in the large intestine typically ranges between -150 and -280 mV [39]. During the *in vitro* digestion, sampling of stomach, small intestine and large intestinal digestion (at 0, 24, 48 and 72 h of the colonic incubation) was done using syringes, causing as little disturbance as possible to the bacterial environment. The samples were then stored at -20°C before analysis. All experiments were performed in triplicate. A schematic overview of the *in vitro* gastrointestinal digestion of meat and consecutive analyses are presented in figure 1.

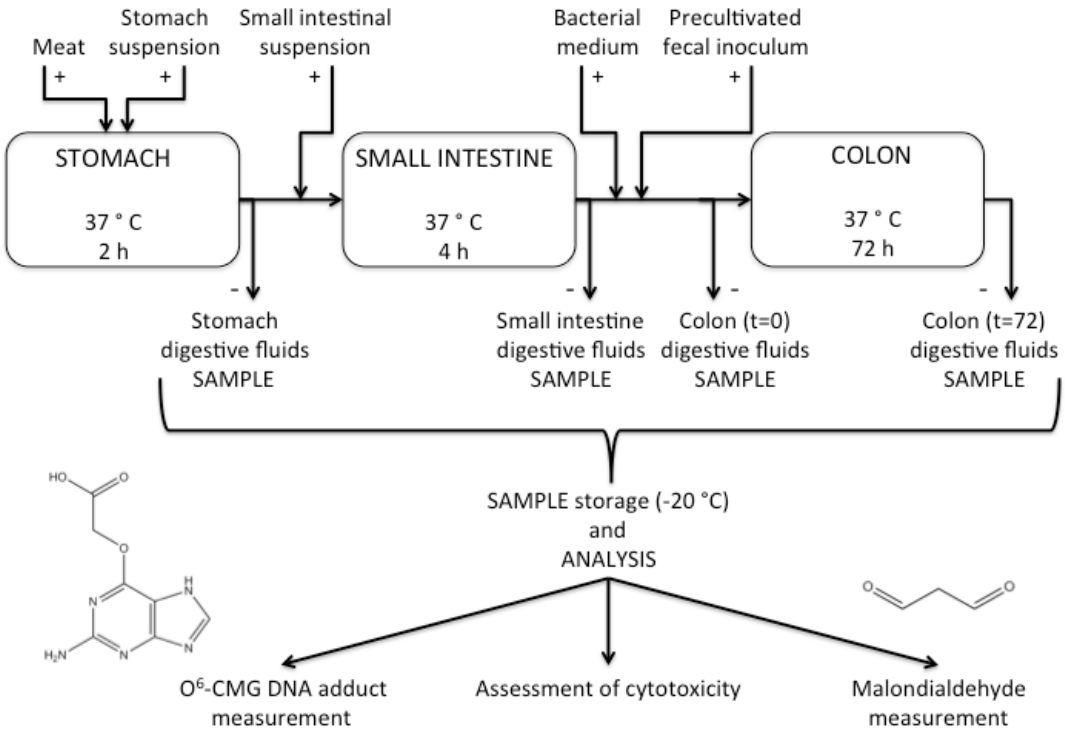

Figure 1. Schematic overview of the experimental setup of the *in vitro* gastrointestinal digestions of meat and consecutive analyses.

2.3 Cell culture

The human colon carcinoma cell line Caco-2 (American Type Culture Collection, Manassas, VA, USA) was cultured as a monolayer in DMEM (Gibco Invitrogen cooperation) containing 10 % fetal calf serum, 1 % non-essential amino acids and penicillin (100 U/mL) and streptomycin (0.1 mg/mL; all from Sigma-Aldrich), at 37°C in a humidified 5 % CO_2 atmosphere. Cells were passaged weekly, using 0.25 % trypsin-EDTA. For the experiments, Caco-2 cells were harvested at 80 % confluency.

2.4 DNA extraction

Caco-2 cells, grown in a monolayer culture (80 % confluency), were collected after a 5 min incubation at 37°C with 0.25 % trypsin-EDTA. Prior to cell lysis, a cell viability rate of 99 % was confirmed by trypan blue exclusion. For DNA extraction, cells were centrifuged for 5 min at 10 000 x g at 4°C to obtain a pellet. Subsequently, 1 mL of DNAzol® reagent (Molecular Research Centre Inc., Cincinnati, OH, USA) was added per 1 x 10^7 of cells. Lysis of the cells was obtained by gently pipetting the mixture or inverting the assay tube. DNA was precipitated from the lysate by adding 0.5 mL of 100 % ethanol per mL of DNAzol® reagent used for the isolation. Next, the sample was mixed by inversion and stored at room temperature for 1–3 min. DNA, visible as a cloudy precipitate, was collected by spooling with a pipette tip, while carefully decanting the supernatant. In case the resulting sheared DNA would not spool, 5 min centrifugation at 5000 x g was applied to obtain a DNA pellet. Finally, the pellet was resuspended in 250–500 µL of TE (Tris-EDTA) buffer. The concentration of the collected DNA was determined by a Nanodrop ND-1000 Spectrophotometer (Isogen Lifescience, IJsselstein, The Netherlands).

2.5 Assessment of cell injury

A colorimetric cell proliferation assay, WST-1 (Roche Diagnostics, Switzerland) was used to assess the cell proliferation and viability of the Caco-2 cells. The tetrazolium salt WST-1 is a ready-to-use substrate that is cleaved by mitochondrial dehydrogenase, which is present in viable cells [40], to formazan dye [41]. The total activity of this mitochondrial dehydrogenase correlates with the number of viable cells, which is in turn quantified by absorbance at 450 nm with a

scanning multi-well spectrophotometer. This is a quick and easy manner to investigate the cytotoxic effect of digestion samples [42].

Caco-2 cells (200 µL; 1.25 e^5 cells/mL) were seeded on flat-bottom 96-well plates and incubated at 37°C, 5 % CO_2 for 24 h. The next day, the DMEM medium was removed and replaced by 100 µL of the digest filtrates diluted in DMEM at 1:10, 1:50 and 1:100. The adhered cells were incubated for 24 h with the digestive fluid. Afterwards, 10 µL of the ready-to-use WST-1 reagent was added to each well and incubated for 2 h at 37°C, UV-VIS absorption was measured at 450 nm. Cell cytotoxicity was determined in quadruplicate and calculated accordingly: $[((A_{Negative\ control} - A_{Blank\ control}) - (A_{Experimental\ value} - A_{Blank\ control}))/(A_{Negative\ control} - A_{Blank\ control})] \times 100$; where $A_{Experimental\ value}$ is the absorbance of the sample, $A_{Blank\ control}$ is the absorbance of DMEM medium and $A_{Negative\ control}$ is the absorbance of cells incubated with DMEM medium. Additionally, some wells were treated with 10 % Triton X-100 solution as a positive control (no cell viability).

For the WST-1 test, a sample selection was made based on the results of the chemical analysis of the NOC-related DNA adducts. To this end, the cytotoxicity of the colonic digestive samples was investigated. In total, six fecal inocula were selected, of which three that displayed DNA adduct formation during colonic digestion and three with no to low DNA adduct concentrations upon colonic digestion.

2.6 Chemical analysis

2.6.1 Extraction protocol for DNA adducts

As a preparative step, all digestive samples were filtersterilized (0.22 m) to ensure the absence of bacterial cells. Afterwards, 182 µL of the filtersterilized sample was supplemented with 100 µg Caco-2 DNA and 5.95 pmol of O^6-Me-d_3-G (internal standard at 118.9 fM). Prior to the incubation step (18 h at 37°C, stirred at a constant speed of 150 RPM), the volume of the samples was standardized to 500 µL by means of TE buffer. The next day, an acidic hydrolysis (2 mL of 0.1 M formic acid for 30 min) at 80°C was performed on all samples to release DNA bases, allowing detection of the individual targeted DNA adducts. Before applying the hydrolysate to a SPE cartridge which was conditioned with 2 mL of 100 % methanol and equilibrated with 2 mL deionized water, it was cooled down in crushed ice. The Oasis HLB SPE (30 mg, 1 mL) was, after loading the hydrolysate, eluted with 2 mL of 100 % methanol. The

collected fraction was then evaporated to dryness (90 min, 20°C) using a SpeedVac® Plus (Savant, Holbrook, NY, USA). Finally, the dried residue was redissolved in a total volume of 100 µL of mobile phase consisting of 90/10 0.05 % acetic acid in deionized water/methanol [31]. As positive and negative control, 100 µg Caco-2 DNA in TE buffer was dissolved and incubated with and without the addition of 2 mM KDA, respectively.

2.6.2 HPLC-MS/MS measurement of DNA adducts

An extensive validation of the LC-MS/MS analysis method for the detection of the NOC-related DNA adducts O^6-MeG and O^6-CMG, characterised with an LOD of 30 and 50 fmol/mg DNA, respectively, as well as the selection of a proper internal standard was previously described by Vanden Bussche et al. [31].

Before every analysis, injection of a standard mixture of the target compounds checked the operational conditions of the chromatographic device. Concentration of the analytes was calculated by fitting area ratios in a 12-point calibration curve, established in Caco-2 DNA fortified with O^6-MeG and O^6-CMG in the range of 0–817 and 0–645 pmol/mg DNA, respectively, with the IS at 118.9 fM. Instrument control and data processing were carried out with Xcalibur Software (Thermo Electron, San José, USA).

2.6.3 Apparent total N-nitroso compound analysis

The digestive samples were analysed by thermal energy analysis for the determination of the apparent total N-nitroso compounds (ATNCs). A selective chemical de-nitrosation with iodine/iodide reagent was performed to detect the ATNCs according to Kuhnle et al. [43]. A digestive homogenate of 100 µL was taken and incubated with 500 µL of aqueous sulphanilamide (50 mg/mL in 1 M HCl) for 5 min to remove unbound nitrite. Afterwards, the sample was injected into a custom-made purge-vessel containing the iodine/iodide reagent kept at 60°C. The released NO was transferred by nitrogen to the NOA chemiluminescence analyser (Model 88 et, Eco Physics, Duernten, Switzerland), *via* a condenser consisting of a NaOH (1 M) trap. For differentiation between the N-nitroso, nitrosothiol and nitroso-heme compounds, mercury (II) stable (nitrosothiols) and potassium ferricyanide stable (nitrosyl iron) compounds

were determined under exactly the same way as described above after an additional incubation with $HgCl_2$ (53 mM) and $K_3Fe(CN)_6$ (4 mM), respectively. Results were expressed as mmol of NO released per L of digestive sample.

2.6.4 TBARS assay

The MDA concentration in the digestive samples was measured by a modified method in accordance with Grotto et al. [35]. TBARS were formed from the reaction of MDA with 2-thiobarbituric acid in an acid environment. The absorbance of the coloured complex was measured spectrophotometrically (Genesis 8 UV-VIS Spectrometer, Spectronic-instruments, UK) at 532 nm with an LOD of 0.05 nmol/mL of digestive sample. The TBARS concentration was expressed in nmole MDA per mL of digestive fluid and determined by means of a 7-point calibration curve with 1,1,3,3-tetramethoxypropane (0–50 nmol/mL).

2.6.5 Metabolic activity

Liquid digestion samples (2 mL) were collected and frozen at -20°C to inhibit further bacterial activity. For analyzing short chain fatty acids (SCFA), the samples were extracted with diethyl ether prior to the analysis on a Di200 gas chromatograph (Shimadzu, Hertogenbosch, The Netherlands). The GC was equipped with a capillary free fatty acid packed column (EC-1000 Econo-Cap column, Alltech, Laarne, Belgium; 25 m x 0.53 mm, film thickness 1.2 m), a flame ionisation detector and a Delsi Nermag 31 integrator (Thermo Separation Products, Wilrijk, Belgium). Nitrogen was used as carrier gas at a flow rate of 20 mL/min. The column temperature and the temperature of the injector and detector were set at 130°C and 195°C, respectively.

For the analysis of phenolic compounds (indol, phenol and *p*-cresol), a liquid/liquid extraction with *n*-hexane was performed prior to the GC-MS detection (PolarisQ, Thermo Fischer Scientific, San Jose, CA, USA). The Trace GC 2000 Gas Chromatograph was fitted with a PolarisQ quadrupole ion trap mass spectrometer. Helium (99.99 % purity, Air Liquide, France) was used as carrier gas at a flow rate of 3 mL/min. A volume of 1 µL was injected (split flow 10 mL/min, splitless time 1 min). The GC was equipped with a Bpx-5 column (SGE, Victoria, Australia; 25 m x 0.22 mm, film thickness 0.25 m), injector, ion source and transfer line

temperature were, respectively, 290°C, 260°C and 250°C. The column temperature consisted of a three-step temperature gradient starting at 40°C and went up to 350°C. Ammonia analysis was performed on a 1026 Kjeltec Auto Distillation apparatus (FOSS Benelux, Amersfoort, The Netherlands). Ammonium in the sample was liberated as ammonia by the addition of MgO. The released ammonia was distilled from the sample into a boric acid solution, which was subsequently back-titrated using a 665 Dosimat (Metrohm, Berchem, Belgium) and 686 Titroprocessor (Metrohm).

2.7 Statistics

Linear mixed model analysis (TIBCO Spotfire S+R 8.2, Göteborg, Sweden) provided a powerful and flexible approach to handle the correlated data (i.e. repeated measurements on each fecal inoculum over time, etc.). In this model, both fixed as well as random effects were incorporated, respectively, the meat type and the variability between the different volunteers. Mixed models can also be extended to non-normal outcomes. Additionally, a paired t-test and an ANOVA fixed effect model (TIBCO Spotfire S+R 8.2) were applied to investigate the individual response variable per volunteer.

3. RESULTS

To investigate the influence of different meat types on specific metabolic and biological end points relevant to the gastrointestinal health status, *in vitro* digestive simulations were performed. Three different meat types (chicken, pork and beef) varying in heme content were selected, with the main focus on beef and chicken. To obtain representative microbial communities during the colonic *in vitro* digestion, 15 fecal inocula, obtained from healthy volunteers, were used in separate fermentation experiments.

3.1 Heme content of meat

The heme contents of the different meat types were determined at 2.57 ± 0.08, 4.97 ± 0.11 and 34.92 ± 0.35 nmol/g for chicken, pork and beef, respectively. Based on the heme analysis of the different meat types, the following heme concentrations were initially present in the incubation flasks: 1.40, 0.20, 0.10 nmol/mL for beef, pork and chicken, respectively.

3.2 NOC-related DNA adducts

The digestive fluids obtained during the different stages of the *in vitro* digestion were incubated with the extracted DNA of the colonic epithelial Caco-2 cell line to investigate the potential formation of O^6-CMG and O^6-MeG. After incubation and extraction, the samples were quantitatively analyzed for the presence of the O^6-CMG and O^6-MeG DNA adducts by LC-MS/MS. As a positive control, Caco-2 DNA was incubated with 2 mM KDA, a reactive intermediate derived from nitrosated glycine, capable of inducing alkylation induced DNA adducts like O^6-CMG and O^6-MeG [15-17]. This resulted in the detection of O^6-CMG at 10.4 ± 1.1 pmol/mL and of O^6-MeG at 1.4 ± 0.3 pmol/mL. Omitting KDA resulted in the absence of both DNA adducts.

O^6-CMG and O^6-MeG could not be detected in the simulated digestive fluids of the stomach and small intestine after overnight incubation with Caco-2 DNA. In samples taken at the end of the colonic digestion (after 72 h of incubation), seven out of 15 colonic digests contained the possibly NOC-derived DNA adduct O^6-CMG. The two positive female inocula and five positive male inocula all belonged to different age categories. At the start of the colonic digestion (0 h), five out of seven digests that were positive at 72 h, already contained low amounts (<55 pmol/mL) of O^6-CMG (table 1). The O^6-CMG concentration in the positive digestive fluid samples increased significantly ($p < 0.05$) from 0 to 72 h for six fecal inocula, whereas one inoculum showed a borderline significant decrease ($p = 0.094$).

Table 1. Mean (± s.e.) concentrations of O^6-CMG (pmol/mL) formed during *in vitro* colonic digestive simulation of chicken and beef by means of the fecal inocula of 15 different individuals (N.D.: not detected; significantly higher with a *p-value < 0.10 or with a **p-value < 0.05).

ID	Sampling time (h)	O^6-CMG (pmol/mL) in chicken	O^6-CMG (pmol/mL) in beef
1	0	33.7 ± 1.2	41.1 ± 4.5
	72	226.4 ± 6.5	539.3 ± 77.5*
2	0	N.D.	N.D.
	72	548.0 ± 33.2	1594.8 ± 119.2**
3	0	9.7 ± 0.5	14.0 ± 0.3**
	72	174.0 ± 10.9	231 ± 14.1**
4-6	0	N.D.	N.D.
	72	N.D.	N.D.
7	0	29.9 ± 1.2	32.3 ± 2.1
	72	619.8 ± 21.7*	492.3 ± 25.6
8	0	50.6 ± 2.0	54.4 ± 4.9
	72	1324.4 ± 103.4	1254.8 ± 75.1
9	0	N.D.	N.D.
	72	37.7 ± 0.8	39.8 ± 1.7
10	0	N.D.	N.D.
	72	46.7 ± 1.3	39.6 ± 3.9
11-15	0	N.D.	N.D.
	72	N.D.	N.D.

Upon linear mixed effect modelling of means, a borderline significant difference ($p = 0.055$) was observed in O^6-CMG DNA adduct formation between the different digested meats at the end of the colonic digestion. When looking at each of the seven O^6-CMG positive fecal inocula individually (by means of paired t-test), the beef indicated a significantly higher genotoxic effect compared to the digested chicken ($p < 0.05$) for two out of seven inocula. The inoculum of one volunteer displayed a borderline significantly higher genotoxic effect for beef ($p < 0.1$; table 1). For one other inoculum, chicken proved to generate more DNA adducts compared to beef ($p < 0.1$; table 1). Noteworthy was that O^6-MeG was never detected in any of the digestive fluids of the 15 volunteers.

During a second experiment, additional digestions of pork (cfr. cytotoxicity of meat) with six different fecal inocula were performed. To this purpose, three O^6-CMG-producing and three non-O^6-CMG-producing fecal inocula were randomly selected. In figure 2, the O^6-CMG concentrations of the three (out of six selected) fecal inocula, which produced the alkylation induced DNA adduct upon colonic digestion, are displayed. Two of these showed a significant difference ($p < 0.05$) between the generated O^6-CMG adducts derived from the digested pork and chicken, whereas the third volunteer only displayed a borderline significant difference ($p < 0.1$). The differences between the digested pork and beef were less pronounced ($p < 0.1$, only for two out of three inocula; figure 2).

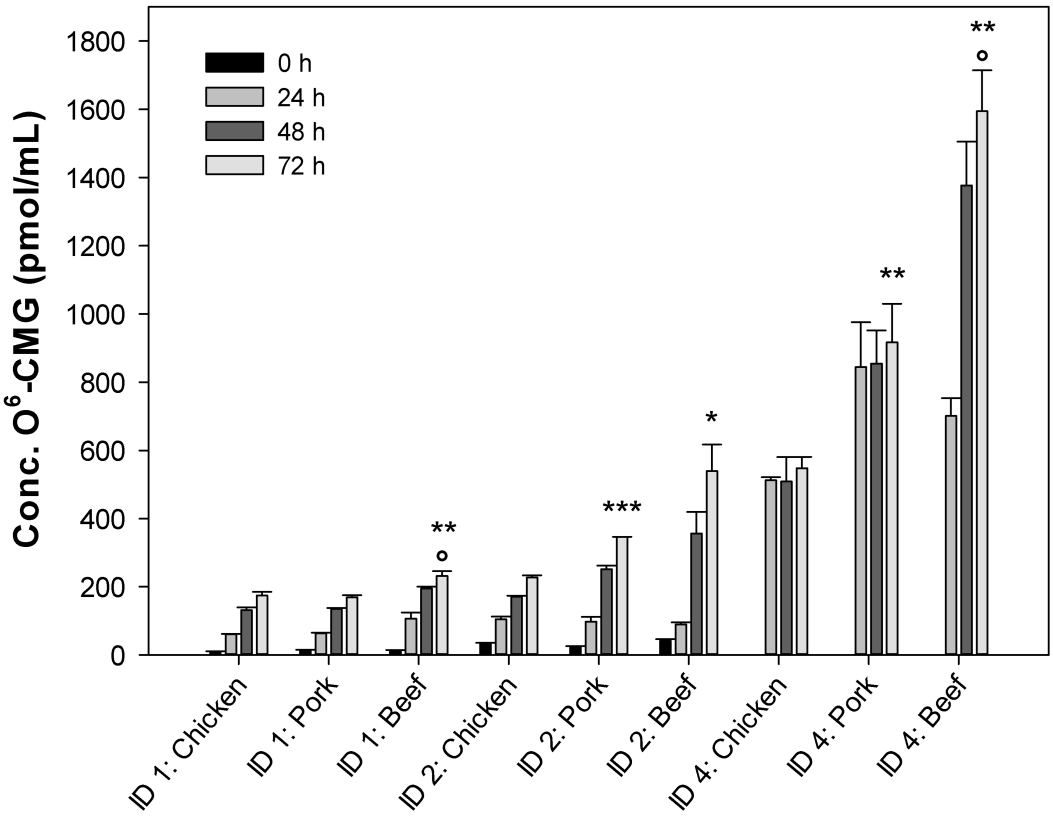

Figure 2. Mean (± s.e.) O^6-CMG levels (pmol/mL) in the different colonic digestive samples (0, 24, 48, 72 h) for three different fecal inocula, performed in triplicate with three different meat types (Significantly higher than chicken with *p-value < 0.1 or **p-value < 0.05 or ***p-value < 0.01; significantly higher than pork with °p-value < 0.1.).

Additionally, an investigation into the involvement of the colonic microbiota in the DNA adduct formation process was envisaged. To this extent, the six selected fecal inocula underwent autoclavation (121°C, 1 atm, 15 min) and *in vitro* digestive simulation of the different meat types. During this experimental set-up, it was not possible to detect O^6-CMG or O^6-MeG in any of the different digestive samples (data not shown).

The addition of a fecal inoculum to the colonic stage of the *in vitro* digestion results in the presence of a large quantity of bacterial DNA, 7–8 log 10 colony forming units/mL digestive fluids. This bacterial DNA might undergo alkylation due to ongoing N-nitrosation taking place during the digestive process. In order to investigate the contribution of bacterial DNA to the formation of the O^6-CMG DNA adduct, the addition of Caco-2 DNA to the incubation step of the digestive samples upon LC-MS/MS analysis was omitted. No significant differences ($p > 0.05$) in DNA adduct levels were observed in the presence or absence of Caco-2 DNA.

3.3 Cytotoxicity of digested meat

The WST-1 proliferation assay was used to investigate the cytotoxic effect of the different meat types during *in vitro* colonic digestion. The digested meat samples were diluted 1:10, 1:50 and 1:100. The latter two displayed only a small decrease in total cell activity (14.1 ± 0.6 %) compared to the control. The 1:10 dilution affected the enzymatic cell activity the most (25.3 ± 1.4 %). At the beginning of the colonic digestion (0 h), a limited but significantly higher cytotoxic effect could be observed for the digested chicken as compared to the beef ($p = 0.053$). For the digested beef samples, a non-significant increase in cytotoxicity ($p > 0.1$) was observed after colonic digestion (0 versus 72 h), in all six of the selected fecal inocula (figure 3).

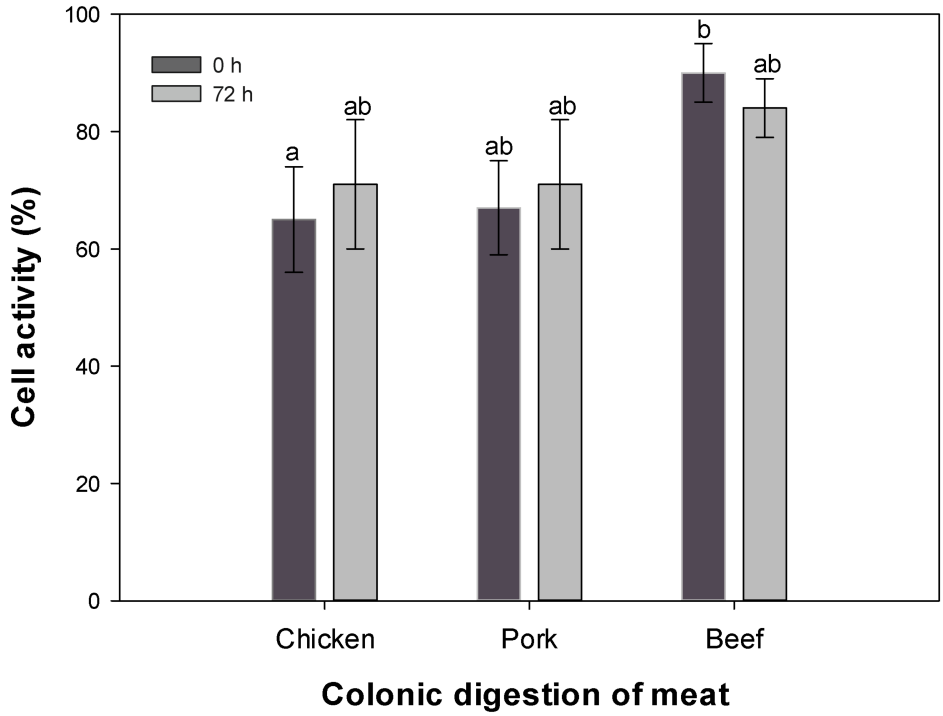

Figure 3. Results of the WST-1 cell proliferation assay of Caco-2 cells incubated (24 h) with the colonic digestive fluids of different meat types (dilution 1:10; 0 and 72 h), calculated as the average of six fecal inocula incubations, each performed in triplicate (a, b: a bar with a different letter differs with a 90 % confidence interval).

3.4 Total apparent N-nitroso compound analysis

The main focus of the ATNC analysis was to single out the NOC fraction from the other NO-contributing compounds, e.g. nitrosothiol, nitrosyl iron, by means of selective chemical denitrosation and chemiluminescence detection [43]. The presence of the NOC-related DNA adduct O^6-CMG was detected during the colonic digestion and peaked towards the end, however no NOCs were found during this stage of the colonic *in vitro* digestion simulation. The only digestive fluids positive for NOCs were the stomach samples, this however at very low concentrations (<35 nmol of NO derived from NOCs detected per L of digestive sample), and with the highest concentration found for the digested beef samples (data not shown).

3.5 TBARS assay

To determine the levels of the main LPO end product MDA in the digestive samples, a TBARS analysis was performed on all digestive fluids. The highest MDA concentrations (15 fecal inocula performed in triplicate, mean ± SE) were observed at the beginning of the colonic simulation (0 h: 22.3 ± 4.9 nmol/mL and 17.5 ± 3.3 nmol MDA/mL in digestive fluids of beef and chicken, respectively) and decreased towards the end (72 h: 10.5 ± 6.1 nmol/mL and 6.9 ± 3.9 nmol MDA/mL for digested beef and chicken, respectively). By mixed linear effect modelling of the means, a significant difference was observed between the MDA results ($p < 0.0001$) between 0 and 72 h of colonic digestion, but also between the different meat types ($p < 0.0004$). As displayed in figure 4, the direction of response for MDA formation appeared to be consistent towards the high-heme meat.

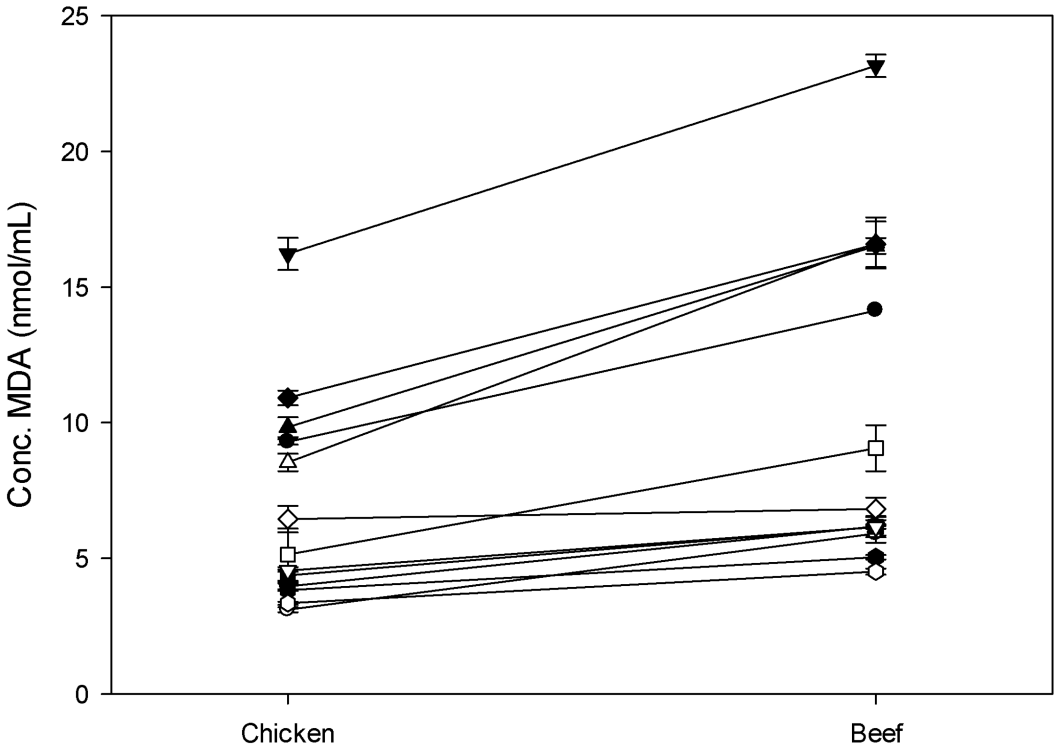

Figure 4. MDA concentrations (mean ± s.e., performed in triplicate) for all 15 volunteers measured at the beginning of the *in vitro* colonic digestive simulation (0 h) for a low and high heme containing meat, chicken and beef, respectively.

3.6 Heme involvement

It is speculated by Corpet [13] that heme iron could play a major role in cancer promotion [44]. This hypothesis is supported by a meta-analysis of epidemiological studies that shows a suggestive association between dietary heme and risk of colon cancer [45]. For this reason, an additional *in vitro* gastrointestinal digestive simulation comprising a sequential stomach, small intestinal and large intestinal digestion was performed. For the large intestinal digestion, a fecal inoculum of an O^6-CMG- and high-MDA-producing volunteer was selected and added. Different amounts of myoglobin, the major heme-containing protein in mammalian muscle tissue, were added at the start (i.e. stomach) of the *in vitro* gastrointestinal beef simulation, 0, 2.8, 14.1 and 28.3 nmol of myoglobin per mL of digestive fluid were supplemented, respectively. As depicted in table 2, a clear positive dose response could be noticed for the O^6-CMG DNA adduct formation when heme-containing myoglobin was added to the digestion. An increase in the myoglobin dose (28.3 nmol/mL) resulted in significant higher concentration levels of O^6-CMG ($p = 0.004$, by ANOVA).

All of the digestive samples of this experiment were also analyzed for their MDA levels, which peaked at 0 h of the colonic digestion. A positive dose response of MDA towards higher myoglobin concentration levels was also observed, with the most pronounced effect at the beginning (0 h) of the colonic digestion ($p = 0.008$) compared to the end (72 h) ($p = 0.015$).

Table 2. Mean (± s.e.) O^6-CMG and MDA concentrations after the addition of different amounts of myoglobin to *in vitro* digestive simulations of beef (N.D.: not detected; ^{a,b} : means within a row with a different subscript differ significantly (p-value < 0.05)).

	Sampling time (h)	Amount of myoglobin added (nmol/mL digestive fluids)			
		0.0	2.8	14.1	28.3
O^6-CMG (pmol/mL)	0	N.D.	N.D.	N.D.	N.D.
	72	2.3 ± 0.7^{a}	7.8 ± 1.2^{a}	12.4 ± 0.6^{a}	36.4 ± 7.5^{b}
MDA (nmol/mL)	0	15.8 ± 0.6^{a}	17.4 ± 0.9^{a}	19.7 ± 1.0^{ab}	19.8 ± 0.5^{b}
	72	6.0 ± 0.1^{a}	7.6 ± 0.7^{ab}	7.8 ± 0.3^{ab}	8.4 ± 0.2^{b}

3.7 Metabolic activity

To evaluate the microbial fermentation during the three different meat incubations (bovine, porcine and poultry), SCFAs, indol, *p*-cresol, phenol and ammonia were monitored during the *in vitro* colonic digestive simulation (table 3 and 4). An increasing amount of SCFAs was produced during the colonic digestion approximating the acetic acid:propionic acid:butyric acid molar ratio of 60:20:20 as put forward by Cummings [46]. The digestion of the different meat types did not influence the SCFA profile significantly, the fecal inocula of the different volunteers however did as shown by the beef data (table 3 and 4). Similar results were obtained for the other meat types. Noteworthy is that elevated butyric acid profiles were observed for three out of six fecal inocula (four to six) at the end of the colonic digestion. The measured concentrations of indol, phenol, *p*-cresol and ammonia did not display significant differences between the different meat types and the different fecal inocula (data not shown).

Table 3. Mean (± s.e.) SCFA concentrations (mM) of the colonic suspensions of the beef digests for the fecal inocula of six selected volunteers (numbered 1 to 6) at 0 h of the colonic digestion (N.D. = not detected; [a, b, c, d, e]: means within a row with a different subscript differ ($p < 0.05$)).

ID	1	2	3	4	5	6
Acetic acid	10.5 ± 0.2^{a}	11.5 ± 1.6^{ab}	3.7 ± 0.4^{c}	14.0 ± 0.1^{bd}	14.3 ± 0.4^{bd}	11.4 ± 0.8^{abd}
Propionic acid	1.0 ± 0.0^{a}	2.5 ± 0.2^{a}	1.7 ± 0.2^{a}	7.9 ± 0.1^{b}	6.9 ± 0.4^{c}	2.0 ± 0.1^{a}
Butyric acid	0.3 ± 0.0^{a}	1.3 ± 0.8^{ab}	8.2 ± 0.7^{c}	23.3 ± 0.7^{d}	10.5 ± 0.8^{c}	2.6 ± 0.2^{b}
Branched acids	N.D.	N.D.	N.D.	0.3 ± 0.0	N.D.	N.D.

Table 4. Mean (± s.e.) SCFA concentrations (mM) of the colonic suspensions of the beef digests for the fecal inocula of six selected volunteers (numbered 1 to 6) at 72 h of the colonic digestion ([a, b, c, d, e]: means within a row with a different subscript differ ($p < 0.05$)).

ID	1	2	3	4	5	6
Acetic acid	52.7 ± 2.3^{abde}	46.4 ± 2.1^{ab}	34.9 ± 2.2^{c}	21.9 ± 0.4^{ad}	61.2 ± 1.7^{abde}	51.0 ± 3.0^{ae}
Propionic acid	9.2 ± 2.2^{a}	22.2 ± 1.5^{b}	20.6 ± 2.7^{ab}	20.1 ± 0.6^{b}	23.8 ± 1.2^{b}	23.6 ± 1.5^{ab}
Butyric acid	21.7 ± 2.6^{a}	12.1 ± 6.0^{abd}	16.6 ± 3.7^{cd}	45.0 ± 1.8^{bcd}	41.6 ± 2.7^{abcde}	32.2 ± 1.0^{ab}
Branched acids	13.1 ± 0.5^{a}	3.2 ± 2.7^{bc}	1.7 ± 0.6^{bc}	0.6 ± 0.1^{abd}	13.7 ± 0.9^{b}	2.3 ± 0.3^{bc}

4. DISCUSSION

It has been suggested that heme iron may play a major role in CRC promotion. Two independent pathways have been hypothesised in explaining the link between heme and CRC promotion. Lipid peroxidation, on the one hand, resulting in the formation of potent aldehydes (i.e. MDA), capable of producing mutagenic DNA adducts, and the N-nitroso pathway, on the other hand, leading to DNA alkylation and DNA adduct formation [13]. The aim of this study was to explore both pathways in order to assess the potential genotoxicity and molecular mechanisms involved in different meat types varying in heme content (beef, chicken and pork) under simulated GIT conditions. To investigate the genotoxicity of the digested meat types, in terms of possible NOC-derived DNA adduct formation, two alkylation type DNA adducts were selected, namely O^6-CMG and O^6-MeG. Previous research has demonstrated their presence in colonic cell tissue, indicating their relevance in CRC promotion [20, 21]. To investigate the NOC-related DNA adduct formation potential of the different digestive fluids, the latter were incubated with extracted DNA of the human colorectal adenocarcinoma cell line Caco-2 prior to LC-MS/MS analysis. This approach was preferred over the incubation with whole cells, which displayed unacceptable inter-batch variation between the different passages of the cell cultures and a low sample throughput [31]. Furthermore, in previous research, we were able to demonstrate that DNA adduct concentrations did not significantly differ if a known NOC (KDA) was added to Caco-2 cells or to pre-extracted Caco-2 DNA [31].

The incubation results demonstrated that the O^6-CMG DNA adduct was formed for seven out of 15 fecal inocula during the large intestinal (colonic) simulation and significantly increased towards the end of the colonic digestion ($p < 0.05$).

When assessing the role of the different GIT compartments in the DNA alkylation pathway, the data of the ATNC measurements with nitrosyl iron as mean contributor and O^6-CMG, both peaking at the end of the colonic digestion, reflect the significance of the large intestine. The key factor in the endogenous alkylation appears to be the presence of an active microbial community, which was acknowledged by the absence of O^6-CMG when autoclaved fecal inocula were added to the batch cultures. Massay et al. [47] reported the same observation in germ-free rats, where N-nitrosation did not occur due to the absence of a normal microbial population. Moreover, the essential role of the individual microbiota is reflected by the observed inter-individual differences [48] in the formation of the O^6-CMG DNA adduct (table 1). It is clear from literature that the

composition and metabolic activity of the colonic microbiota can differ considerably between and even in individuals during the course of their lifetime [48]. With respect to NOC formation, the N-nitrosating properties of certain bacteria colonizing the individual human gut (and a possible connection to carcinogenesis) have been demonstrated before [49-51]. Furthermore, the possible role (of the exact composition) of the individual gut microbiota in health and disease was already pointed out decades ago [52], and has been brought to the surface even more in recent years [53, 54]. Other examples of significant inter-individual differences in the transformation of dietary compounds or contaminants such as, phytoestrogens [55], heterocyclic aromatic amines [56], polycyclic aromatic hydrocarbons [37], etc. have been reported.

For five out of seven O^6-CMG-generating fecal inocula, the O^6-CMG DNA adduct could already be detected at the mere beginning of the colonic digestion. The only difference between the small intestinal fluids, where O^6-CMG was never detected, and the initial colonic digestive fluids (before 72 h of incubation), is the addition of the individual fecal inoculum. We concluded that for those five volunteers, O^6-CMG was already present in the fecal inoculum, most probably due to *in vivo* formation of this DNA adduct prior to sampling. O^6-CMG quantities in the fecal inocula of the two other volunteers may have been absent or below the detection limit of the utilized LC-MS/MS method. Indeed, the presence of O^6-CMG in certain human fecal samples further demonstrates the *in vivo* relevance of O^6-CMG since O^6-CMG has previously been detected in various biological samples (i.e. blood, colonic biopsies and exfoliated colonocytes) [17, 20, 21, 31].

To investigate the genotoxicity of the different meat types (i.e. chicken and beef) with respect to the NOC pathway, a mixed model analysis of the entire dataset was performed. This statistical analysis demonstrated a borderline significantly higher ($p = 0.055$) O^6-CMG concentration for beef versus chicken at the end of the colonic digestion (72 h). The suggested higher genotoxicity of red meat is in line with previous *in vivo* studies, indicating that a higher N-nitrosation occurs when heme-rich meat has been digested [7, 8, 21, 43]. Most of these studies used the fecal ATNC concentration to compare the dose response of heme-rich versus low-heme meat. Accordingly, ATNC analyses were performed on the digestive samples obtained from our *in vitro* stomach, small and large intestinal digestions to correlate the N-nitrosation process during the incubations with the obtained O^6-CMG values. No NOCs were detected in the digestive fluids of the small and large intestinal digestion. This finding might also suggest as reported by Mirvish [18] that besides NOCs, other nitrite-derived alkylating agents may be responsible for the

formation of O^6-CMG. Moreover, these agents might be unable to form O^6-MeG, providing a potential additional explanation for the absence of O^6-MeG. The largest fraction of the ATNC measured (data not shown) however consisted of nitrosyl iron, reinforcing the involvement of heme in the link between a high red meat diet and CRC [11, 13, 21, 43].

To mimic the human *in vivo* gastrointestinal digestion of meat, this study relied on sequential *in vitro* incubations. These *in vitro* models take into account the human physiology by simulating the transit through the human digestive tract, which can be executed either by using separate GI compartments or by sequential exposure of the food source to simulated mouth, gastric, small and large intestinal conditions [57-59]. Though the use of these types of batch cultures has its limitations, i.e. absence of gastrointestinal absorption, lack of interaction with the host colonic mucosa, etc., *in vivo* studies were not considered here due to lack of versatility in terms of mechanistic explorative work as well as time consuming and costly nature [60]. Furthermore, whenever possible, *in vivo* studies should be avoided due to ethical considerations. For this study, the advantage of sequential batch cultures lies with the fact that they allow to investigate which element or mechanism within the gastrointestinal digestion is involved in the studied process. To enable the use of different fecal inocula in a shorter time frame in combination with a consecutive multi-phase simulation of the GIT, static over dynamic batch cultures were preferred [57].

The addition of pre-cultured microbiota (fecal inoculum) to the batch cultures resulted in the presence of a large quantity of bacterial DNA (350 pmol DNA/mL digestive fluids for 7–8 log 10 colony forming units/mL). Analyzing the colonic digestive samples for O^6-CMG without the incubation of Caco-2 DNA did not result in a significant difference in O^6-CMG levels. This finding suggests that alkylation of the DNA occurred during the *in vitro* digestion, indicating the highly reactive nature of either NOCs, possibly explaining the absence of NOCs in the ATNC measurements of the colonic digestive fluids [61] or of other nitrite-derived alkylating agents. Under *in vivo* circumstances, alkylating agents that are produced in the colon will most likely not only bind to bacterial DNA, but through the intimate contact that exists between the microbiota and the colonic epithelium [62], also to human DNA, and result in the formation of DNA adducts. As a result, the measurement of the O^6-CMG DNA adducts, although originating from bacterial or fecal exfoliate DNA in this study, is considered as a biomarker for potential DNA adduct formation *in vivo*.

Previously reported observations [15, 16] indicate that carboxymethylation at the O^6 atom of 2'-deoxyguanosine appeared to be a common feature when nitrosated glycine derivatives are formed. The reported concomitant methylation however appeared absent in our digestive simulations, and no O^6-MeG adducts could be detected in the digestive fluids. O^6-MeG can be repaired under *in vitro* circumstances by MGMT [15, 63], a repair mechanism expressed by the Caco-2 cell line [64]. Nevertheless, in our experiments, pre-extracted Caco-2 DNA was used, which is most likely depleted of any MGMT molecules as a result of the DNA extraction procedure [31]. Therefore, it is believed that MGMT could not have been responsible for the absence of O^6-MeG. Indeed, in literature it has been consistently reported that the formation rate of O^6-MeG compared to O^6-CMG is significantly lower when incubating DNA with KDA [15, 16, 31], which could explain its absence. As for O^6-CMG, only very recently a report was published of its ability to act as a MGMT substrate in synthetic oligodeoxyribonucleotides [65]. This finding could possibly undermine O^6-CMG biomarker capacities for CRC. However, in a previously reported study by Vanden Bussche et al. [31], Caco-2 cells as well as the pre-extracted DNA thereof were incubated with KDA and similar levels of O^6-CMG DNA adducts were formed, which might suggest that the MGMT repair system is not potent enough to repair a considerable amount of O^6-CMG adducts. Based on this finding and the presence of O^6-CMG in biological samples [20, 21, 31], it would still seem acceptable to consider O^6-CMG as an adequate biomarker and potential causative agent for CRC.

Next to the alkylation pathway, the peroxidation of polyunsaturated fatty acids with MDA as predominant product has been suggested by Corpet [13], as an additional mechanistic hypothesis for the existing link between dietary heme intake and CRC risk. The MDA measurements demonstrated significant differences ($p = 0.0004$) between the digestion of beef and chicken, suggesting the higher genotoxic potential of red meat consumption, with the highest levels generated before colonic digestion (table 2). These MDA levels may be correlated to the data of the WST-1 cell proliferation assay where only at the beginning of the colonic digestion a borderline significant difference was observed between the digested beef and chicken ($p = 0.053$). The latter effect disappeared towards the end of the colonic digestion, which could be linked to the decline in MDA level. However, it needs to be stated that the results of the cytotoxicity test proved inconclusive and tests based on different mechanisms, e.g. detection of caspase positive cells (marker for apoptosis) [66], checking membrane permeability by propidium iodides staining [67], might be advisable for future experiments. As for the significant MDA decrease during

colonic digestion, this could be attributed to different factors: i.e. degradation into volatile compounds, formation of Schiff bases through reaction with protein chains, reaction with bacterial DNA resulting in several DNA adducts (e.g. 3-(2-deoxy--D-erythro-pentofuranosyl)pyrimido[1,2-]purin-10(3H)-one (M_1dG) [23]), oxidation by bacterial aldehyde dehydrogenase activity [68] and presence of dietary fibre in the fecal inocula, which are known to bind MDA [69].

An additional incubation experiment was performed to substantiate the heme iron hypothesis as potential causative CRC agent [13] by adding myoglobin to the *in vitro* digestion. Both O^6-CMG and MDA increased significantly upon addition of 28.3 nmol myoglobin per mL of digestive fluid. These results indicate the involvement of the myoglobin protein and heme molecule, which are more present in red than white meat, in the active formation of O^6-CMG and MDA. Next to the involvement of heme in the gastrointestinal formation of cyto- and genotoxic compounds upon red meat digestion, it is very likely that other factors may play a role in the formation pathway(s) of both MDA and the O^6-CMG DNA adduct. As a first factor, the colonic microbiota consist of a complex mixture with several attributes that differ inter- and intra-individually and even by anatomical site along the colon and their location within the lumen [48, 54]. The colonic bacteria are constantly influenced by their surroundings; heme-Fe can, e.g. increase the prevalence of *Enterobacteria* and *Bacteroidetes* spp. and decrease *Lactobacilli* and *Firmicutes* spp. [70]. Additionally, it has been reported that certain *Lactobaccilli* and *Bifidobacteria* spp. are capable of producing nitric oxide (• NO) from nitrite [71]. Depending on the •NO to reactive oxygen species ratio, •NO can enhance or inhibit oxidation processes [72] and thus MDA formation. Of course, nitrite and •NO may play their part in the nitrosation pathway as well. Furthermore, high-fat diets give rise to a decrease in *Bifidobacteria*. *Bifidobacteria* are involved in the production of SCFAs such as butyrate, which is known for its beneficial anti-carcinogenic attributes. Another important and possibly interfering factor that is linked to diet is the remaining amount of polyunsaturated fatty acids (PUFAs) in feces [73] and thus also in the digestive flasks. Since PUFAs initiate the LPO process, variation in PUFA content (both in feces and meat) may reflect directly upon MDA formation. These and other modulating factors in both the O^6-CMG and MDA formation pathway will influence the heme-Fe dose response.

To evaluate whether proper microbial fermentation occurred during the *in vitro* digestion simulation, SCFA levels were monitored, since these are the main end products resulting from colonic bacterial fermentation of dietary carbohydrates. The most important SCFAs are acetic

acid, propionic acid and butyric acid, which occur in molar ratios of about 60:20:20 in the colon [46]. The SCFA profiles of the conducted *in vitro* digestions (table 3 and 4) indicated proper microbial fermentation, approximating the *in vivo* molar ratios. The SCFA profiles obtained from three out of six selected fecal inocula showed a significantly higher production of butyric acid. It is not uncommon that butyric acid increases or even exceeds the levels of propionic acid as a result of peptide supplementation [74], which may be considered as a probable cause since meat is an important protein source. Additionally, some human fecal bacteria are known as net consumers of acetic acid, resulting in the production of butyric acid [75], which could have been the case in our experimental set-up due to the extensive batch culture duration of 72 h. Butyric acid is known to exert important effects on cell differentiation and gut health [76] and is thought to play a protective role against CRC [77]. Indeed, the batch cultures with high butyric acid profiles were those with almost no O^6-CMG formation (<15 pmol/mL). In this case, one could propose a potential butyrogenic effect, although further research would be required to confirm this. The other digestive products, i.e. phenol, indo, *p*-cresol and ammonia did not significantly differ between the different meat types or the fecal inocula.

In this study, two independent pathways have been set forward to mechanistically explain the reported correlation between red meat consumption and CRC, namely the alkylation and LPO pathway. The *in vitro* experiments clearly demonstrated that both pathways depend on different mechanisms, but may both require the presence of myoglobin (representing the meat-specific heme iron fraction) as a catalytic agent. While endogenous alkylation was observed during colonic fermentation, MDA formation peaked during the small intestinal digestion. Moreover, the formation of the alkylated DNA adduct O^6-CMG appeared to depend on the microbial composition, since the inter-individual variability of the fecal inocula influenced the DNA adduct formation considerably and autoclavation completely inhibited the process. A contributing factor in the MDA production was most likely the inherent fat content of the meat [8], since MDA is the main by-product of the peroxidation of polyunsaturated fatty acids, which is known to be prone to iron-mediation [78]. Both factors play a role in the MDA formation, which significantly differed between the digested beef and chicken ($p < 0.0004$).

To conclude, our *in vitro* digestions confirmed that the consumption of high-heme meat (beef) may exert genotoxic effects in the GIT. As for the role of the gastrointestinal digestion, it was demonstrated that the duodenum displayed the highest LPO rate with subsequent MDA formation, while for the alkylated DNA adduct O^6-CMG, the colon and its innate microbial

biota were proven to be vital and the basis for the significant observed variability between the individual fecal inocula.

REFERENCES

[1] T. Norat, S. Bingham, P. Ferrari, N. Slimani, M. Jenab, M. Mazuir, K. Overvad, A. Olsen, A. Tjonneland, F. Clavel, M.C. Boutron-Ruault, E. Kesse, H. Boeing, M.M. Bergmann, A. Nieters, J. Linseisen, A. Trichopoulou, D. Trichopoulos, Y. Tountas, F. Berrino, D. Palli, S. Panico, R. Tumino, P. Vineis, H.B. Bueno-de-Mesquita, P.H. Peeters, D. Engeset, E. Lund, G. Skeie, E. Ardanaz, C. Gonzalez, C. Navarro, J.R. Quiros, M.J. Sanchez, G. Berglund, I. Mattisson, G. Hallmans, R. Palmqvist, N.E. Day, K.T. Khaw, T.J. Key, M. San Joaquin, B. Hemon, R. Saracci, R. Kaaks, E. Riboli, Meat, fish, and colorectal cancer risk: the European Prospective Investigation into cancer and nutrition, Journal of the National Cancer Institute, 97 (2005) 906-916.
[2] R. Micha, S.K. Wallace, D. Mozaffarian, Red and processed meat consumption and risk of incident coronary heart disease, stroke, and diabetes mellitus: a systematic review and meta-analysis, Circulation, 121 (2010) 2271-2283.
[3] A. Pan, Q. Sun, A.M. Bernstein, J.E. Manson, W.C. Willett, F.B. Hu, Changes in red meat consumption and subsequent risk of type 2 diabetes mellitus: Three cohorts of us men and women, JAMA Internal Medicine, 173 (2013) 1328-1335.
[4] S. Mannisto, J. Kontto, M. Kataja-Tuomola, D. Albanes, J. Virtamo, High processed meat consumption is a risk factor of type 2 diabetes in the Alpha-Tocopherol, Beta-Carotene Cancer Prevention study, Br J Nutr, 103 (2010) 1817-1822.
[5] S.C. Larsson, N. Orsini, A. Wolk, Diabetes mellitus and risk of colorectal cancer: a meta-analysis, Journal of the National Cancer Institute, 97 (2005) 1679-1687.
[6] E. Giovannucci, Metabolic syndrome, hyperinsulinemia, and colon cancer: a review, The American journal of clinical nutrition, 86 (2007) s836-842.
[7] S.A. Bingham, B. Pignatelli, J.R. Pollock, A. Ellul, C. Malaveille, G. Gross, S. Runswick, J.H. Cummings, I.K. O'Neill, Does increased endogenous formation of N-nitroso compounds in the human colon explain the association between red meat and colon cancer?, Carcinogenesis, 17 (1996) 515-523.
[8] R. Hughes, A.J. Cross, J.R. Pollock, S. Bingham, Dose-dependent effect of dietary meat on endogenous colonic N-nitrosation, Carcinogenesis, 22 (2001) 199-202.
[9] S.A. Bingham, R. Hughes, A.J. Cross, Effect of White Versus Red Meat on Endogenous N-Nitrosation in the Human Colon and Further Evidence of a Dose Response, The Journal of nutrition, 132 (2002) 3522S-3525S.
[10] G. Lombardi-Boccia, B. Martinez-Dominguez, A. Aguzzi, Total Heme and Non-heme Iron in Raw and Cooked Meats, Journal of Food Science, 67 (2002) 1738-1741.
[11] A.J. Cross, J.R. Pollock, S.A. Bingham, Haem, not protein or inorganic iron, is responsible for endogenous intestinal N-nitrosation arising from red meat, Cancer Res, 63 (2003) 2358-2360.
[12] R. Bonnett, A.A. Charalambides, R.A. Martin, K.D. Sales, B.W. Fitzsimmons, Reactions of nitrous acid and nitric oxide with porphyrins and haems. Nitrosylhaems as nitrosating agents, Journal of the Chemical Society, Chemical Communications, (1975) 884-885.

[13] D.E. Corpet, Red meat and colon cancer: should we become vegetarians, or can we make meat safer?, Meat science, 89 (2011) 310-316.
[14] S.S. Mirvish, Formation of N-nitroso compounds: Chemistry, kinetics, and in vivo occurrence, Toxicology and applied pharmacology, 31 (1975) 325-351.
[15] D.E.G. Shuker, G.P. Margison, Nitrosated Glycine Derivatives as a Potential Source of O6-Methylguanine in DNA, Cancer Research, 57 (1997) 366-369.
[16] K.L. Harrison, R. Jukes, D.P. Cooper, D.E. Shuker, Detection of concomitant formation of O6-carboxymethyl- and O6-methyl-2'-deoxyguanosine in DNA exposed to nitrosated glycine derivatives using a combined immunoaffinity/HPLC method, Chemical research in toxicology, 12 (1999) 106-111.
[17] B.C. Cupid, Z. Zeng, R. Singh, D.E. Shuker, Detection of O6-carboxymethyl-2'-deoxyguanosine in DNA following reaction of nitric oxide with glycine and in human blood DNA using a quantitative immunoslot blot assay, Chemical research in toxicology, 17 (2004) 294-300.
[18] S.S. Mirvish, Role of N-nitroso compounds (NOC) and N-nitrosation in etiology of gastric, esophageal, nasopharyngeal and bladder cancer and contribution to cancer of known exposures to NOC, Cancer letters, 93 (1995) 17-48.
[19] B.C. Challis, Chemistry and biology of nitrosated peptides, Cancer surveys, 8 (1989) 363-384.
[20] C.N. Hall, A.F. Badawi, P.J. O'Connor, R. Saffhill, The detection of alkylation damage in the DNA of human gastrointestinal tissues, British journal of cancer, 64 (1991) 59-63.
[21] M.H. Lewin, N. Bailey, T. Bandaletova, R. Bowman, A.J. Cross, J. Pollock, D.E.G. Shuker, S.A. Bingham, Red Meat Enhances the Colonic Formation of the DNA Adduct O6-Carboxymethyl Guanine: Implications for Colorectal Cancer Risk, Cancer Research, 66 (2006) 1859-1865.
[22] S.A. Moore, O. Xeniou, Z.T. Zeng, E. Humphreys, S. Burr, E. Gottschalg, S.A. Bingham, D.E. Shuker, Optimizing immunoslot blot assays and application to low DNA adduct levels using an amplification approach, Analytical biochemistry, 403 (2010) 67-73.
[23] U. Nair, H. Bartsch, J. Nair, Lipid peroxidation-induced DNA damage in cancer-prone inflammatory diseases: A review of published adduct types and levels in humans, Free Radical Biology and Medicine, 43 (2007) 1109-1120.
[24] D.H. Phillips, V.M. Arlt, (3)(2)P-postlabeling analysis of DNA adducts, Methods in molecular biology (Clifton, N.J.), 1105 (2014) 127-138.
[25] N.J. van Sittert, P.J. Boogaard, A.T. Natarajan, A.D. Tates, L.G. Ehrenberg, M.A. Törnqvist, Formation of DNA adducts and induction of mutagenic effects in rats following 4 weeks inhalation exposure to ethylene oxide as a basis for cancer risk assessment, Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 447 (2000) 27-48.
[26] E. Gottschalg, G.B. Scott, P.A. Burns, D.E. Shuker, Potassium diazoacetate-induced p53 mutations in vitro in relation to formation of O6-carboxymethyl- and O6-methyl-2'-deoxyguanosine DNA adducts: relevance for gastrointestinal cancer, Carcinogenesis, 28 (2007) 356-362.
[27] P.B. Farmer, K. Brown, E. Tompkins, V.L. Emms, D.J. Jones, R. Singh, D.H. Phillips, DNA adducts: mass spectrometry methods and future prospects, Toxicology and applied pharmacology, 207 (2005) 293-301.
[28] R. Singh, P.B. Farmer, Liquid chromatography-electrospray ionization-mass spectrometry: the future of DNA adduct detection, Carcinogenesis, 27 (2006) 178-196.
[29] M.W. Himmelstein, P.J. Boogaard, J. Cadet, P.B. Farmer, J.H. Kim, E.A. Martin, R. Persaud, D.E. Shuker, Creating context for the use of DNA adduct data in cancer risk assessment: II. Overview of methods of identification and quantitation of DNA damage, Critical reviews in toxicology, 39 (2009) 679-694.

[30] F. Zhang, M.J. Bartels, L.H. Pottenger, B.B. Gollapudi, M.R. Schisler, Simultaneous quantitation of 7-methyl- and O6-methylguanine adducts in DNA by liquid chromatography-positive electrospray tandem mass spectrometry, Journal of chromatography. B, Analytical technologies in the biomedical and life sciences, 833 (2006) 141-148.
[31] J. Vanden Bussche, S.A. Moore, F. Pasmans, G.G. Kuhnle, L. Vanhaecke, An approach based on ultra-high pressure liquid chromatography-tandem mass spectrometry to quantify O6-methyl and O6-carboxymethylguanine DNA adducts in intestinal cell lines, Journal of chromatography. A, 1257 (2012) 25-33.
[32] D. Del Rio, A.J. Stewart, N. Pellegrini, A review of recent studies on malondialdehyde as toxic molecule and biological marker of oxidative stress, Nutrition, metabolism, and cardiovascular diseases : NMCD, 15 (2005) 316-328.
[33] E. Niki, Lipid peroxidation: physiological levels and dual biological effects, Free radical biology & medicine, 47 (2009) 469-484.
[34] L.J. Marnett, Oxy radicals, lipid peroxidation and DNA damage, Toxicology, 181-182 (2002) 219-222.
[35] D. Grotto, L.D. Santa Maria, S. Boeira, J. Valentini, M.F. Charão, A.M. Moro, P.C. Nascimento, V.J. Pomblum, S.C. Garcia, Rapid quantification of malondialdehyde in plasma by high performance liquid chromatography–visible detection, Journal of Pharmaceutical and Biomedical Analysis, 43 (2007) 619-624.
[36] J.A. Knight, R.K. Pieper, L. McClellan, Specificity of the thiobarbituric acid reaction: its use in studies of lipid peroxidation, Clinical chemistry, 34 (1988) 2433-2438.
[37] T. Van de Wiele, L. Vanhaecke, C. Boeckaert, K. Peru, J. Headley, W. Verstraete, S. Siciliano, Human colon microbiota transform polycyclic aromatic hydrocarbons to estrogenic metabolites, Environmental health perspectives, 113 (2005) 6-10.
[38] H.C. Hornsey, The color of cooked cured pork. I. Estimation of the nitric oxide-haem pigments., J. Sci. Food Agr., 7 (1956).
[39] C.R. Jonas, C.F. Estívariz, D.P. Jones, L.H. Gu, T.M. Wallace, E.E. Diaz, R.R. Pascal, G.A. Cotsonis, T.R. Ziegler, Keratinocyte Growth Factor Enhances Glutathione Redox State in Rat Intestinal Mucosa during Nutritional Repletion, The Journal of nutrition, 129 (1999) 1278-1284.
[40] M. Ishiyama, H. Tominaga, M. Shiga, K. Sasamoto, Y. Ohkura, K. Ueno, A combined assay of cell viability and in vitro cytotoxicity with a highly water-soluble tetrazolium salt, neutral red and crystal violet, Biol Pharm Bull, 19 (1996) 1518-1520.
[41] T.F. Slater, B. Sawyer, U. Straeuli, Studies on succinate-tetrazolium reductase systems. III. Points of coupling of four different tetrazolium salts., Biochimica et biophysica acta, 77 (1963) 383-393.
[42] M.A. Carlson, Technical note: assay of cell quantity in the fibroblast-populated collagen matrix with a tetrazolium reagent, Eur Cell Mater, 12 (2006) 44-48.
[43] G.G. Kuhnle, G.W. Story, T. Reda, A.R. Mani, K.P. Moore, J.C. Lunn, S.A. Bingham, Diet-induced endogenous formation of nitroso compounds in the GI tract, Free radical biology & medicine, 43 (2007) 1040-1047.
[44] T. Sawa, T. Akaike, K. Kida, Y. Fukushima, K. Takagi, H. Maeda, Lipid peroxyl radicals from oxidized oils and heme-iron: implication of a high-fat diet in colon carcinogenesis, Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology, 7 (1998) 1007-1012.
[45] N.M. Bastide, F.H. Pierre, D.E. Corpet, Heme iron from meat and risk of colorectal cancer: a meta-analysis and a review of the mechanisms involved, Cancer prevention research (Philadelphia, Pa.), 4 (2011) 177-184.
[46] J.H. Cummings, Short chain fatty acids in the human colon, Gut, 22 (1981) 763-779.

[47] R.C. Massey, P.E. Key, A.K. Mallett, I.R. Rowland, An investigation of the endogenous formation of apparent total N-nitroso compounds in conventional microflora and germ-free rats, Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association, 26 (1988) 595-600.
[48] P.J. Turnbaugh, R.E. Ley, M. Hamady, C. Fraser-Liggett, R. Knight, J.I. Gordon, The human microbiome project: exploring the microbial part of ourselves in a changing world, Nature, 449 (2007) 804-810.
[49] S.A. Leach, M. Thompson, M. Hill, Bacterially catalysed N-nitrosation reactions and their relative importance in the human stomach, Carcinogenesis, 8 (1987) 1907-1912.
[50] A. Engemann, C. Focke, H.U. Humpf, Intestinal formation of N-nitroso compounds in the pig cecum model, J Agric Food Chem, 61 (2013) 998-1005.
[51] K. Suzuki, T. Mitsuoka, N-nitrosamine formation by intestinal bacteria, IARC scientific publications, (1984) 275-281.
[52] M.J.E. Hill, The Role of Gut Bacteria in Human Toxicology and Pharmacology, Taylor & Francis, London, (1995).
[53] I. Sekirov, S.L. Russell, L.C. Antunes, B.B. Finlay, Gut microbiota in health and disease, Physiol Rev, 90 (2010) 859-904.
[54] B.S.T. Culpepper, V. Mai, Evidence for Contributions of Gut Microbiota to Colorectal Carcinogenesis, Current Nutrition Reports, 2 (2013) 10-18.
[55] K. Decroos, S. Cattoir, N. Boon, W. Verstraete, In vitro production of equol by human intestinal microbiota, The Journal of nutrition, 134 (2004) 1283S,1284S.
[56] F. Kassie, S. Rabot, M. Kundi, M. Chabicovsky, H.-M. Qin, S. Knasmüller, Intestinal microflora plays a crucial role in the genotoxicity of the cooked food mutagen 2-amino-3-methylimidazo[4,5-f]quinoline (IQ), Carcinogenesis, 22 (2001) 1721-1725.
[57] M. Wickham, R. Faulks, C. Mills, In vitro digestion methods for assessing the effect of food structure on allergen breakdown, Molecular nutrition & food research, 53 (2009) 952-958.
[58] S.J. Hur, B.O. Lim, E.A. Decker, D.J. McClements, In vitro human digestion models for food applications, Food chemistry, 125 (2011) 1-12.
[59] T. Van de Wiele, C.M. Gallawa, K.M. Kubachka, J.T. Creed, N. Basta, E.A. Dayton, S. Whitacre, G. Du Laing, K. Bradham, Arsenic metabolism by human gut microbiota upon in vitro digestion of contaminated soils, Environmental health perspectives, 118 (2010) 1004-1009.
[60] A.G. Oomen, A. Hack, M. Minekus, E. Zeijdner, C. Cornelis, G. Schoeters, W. Verstraete, T. Van de Wiele, J. Wragg, C.J. Rompelberg, A.J. Sips, J.H. Van Wijnen, Comparison of five in vitro digestion models to study the bioaccessibility of soil contaminants, Environ Sci Technol, 36 (2002) 3326-3334.
[61] J.E. Stuff, E.T. Goh, S.L. Barrera, M.L. Bondy, M.R. Forman, Construction of an N-nitroso database for assessing dietary intake, Journal of food composition and analysis : an official publication of the United Nations University, International Network of Food Data Systems, 22 (2009) S42-S47.
[62] M. Manco, L. Putignani, G.F. Bottazzo, Gut microbiota, lipopolysaccharides, and innate immunity in the pathogenesis of obesity and cardiovascular risk, Endocr Rev, 31 (2010) 817-844.
[63] M. O'Driscoll, P. Macpherson, Y.-Z. Xu, P. Karran, The cytotoxicity of DNA carboxymethylation and methylation by the model carboxymethylating agent azaserine in human cells, Carcinogenesis, 20 (1999) 1855-1862.
[64] A.A. Ramos, D.F. Pedro, C.F. Lima, A.R. Collins, C. Pereira-Wilson, Development of a new application of the comet assay to assess levels of O6-methylguanine in genomic DNA (CoMeth), Free radical biology & medicine, 60 (2013) 41-48.
[65] P. Senthong, C.L. Millington, O.J. Wilkinson, A.S. Marriott, A.J. Watson, O. Reamtong, C.E. Eyers, D.M. Williams, G.P. Margison, A.C. Povey, The nitrosated bile acid DNA lesion O6-

carboxymethylguanine is a substrate for the human DNA repair protein O6-methylguanine-DNA methyltransferase, Nucleic Acids Res, 41 (2013) 3047-3055.
[66] K.R. Jerome, D.D. Sloan, M. Aubert, Measurement of CTL-induced cytotoxicity: the caspase 3 assay, Apoptosis, 8 (2003) 563-571.
[67] T.T. Liao, R.W. Jia, Y.L. Shi, J.W. Jia, L. Wang, H. Chua, Propidium iodide staining method for testing the cytotoxicity of 2,4,6-trichlorophenol and perfluorooctane sulfonate at low concentrations with Vero cells, J Environ Sci Health A Tox Hazard Subst Environ Eng, 46 (2011) 1769-1775.
[68] T. Nosova, K. Jokelainen, P. Kaihovaara, H. Jousimies-Somer, A. Siitonen, R. Heine, M. Salaspuro, Aldehyde dehydrogenase activity and acetate production by aerobic bacteria representing the normal flora of human large intestine, Alcohol Alcohol, 31 (1996) 555-564.
[69] S.J. Hur, B.O. Lim, G.B. Park, S.T. Joo, Effects of Various Fiber Additions on Lipid Digestion during In Vitro Digestion of Beef Patties, Journal of Food Science, 74 (2009) C653-C657.
[70] N. IJssennagger, M. Derrien, G.M. van Doorn, A. Rijnierse, B. van den Bogert, M. Muller, J. Dekker, M. Kleerebezem, R. van der Meer, Dietary heme alters microbiota and mucosa of mouse colon without functional changes in host-microbe cross-talk, PloS one, 7 (2012) e49868.
[71] T. Sobko, C.I. Reinders, E. Jansson, E. Norin, T. Midtvedt, J.O. Lundberg, Gastrointestinal bacteria generate nitric oxide from nitrate and nitrite, Nitric Oxide, 13 (2005) 272-278.
[72] V. Darley-Usmar, H. Wiseman, B. Halliwell, Nitric oxide and oxygen radicals: a question of balance, FEBS Lett, 369 (1995) 131-135.
[73] L. Amate, A. Gil, M. Ramírez, Dietary Long-Chain Polyunsaturated Fatty Acids from Different Sources Affect Fat and Fatty Acid Excretions in Rats, The Journal of nutrition, 131 (2001) 3216-3221.
[74] A.W. Walker, S.H. Duncan, E.C. McWilliam Leitch, M.W. Child, H.J. Flint, pH and peptide supply can radically alter bacterial populations and short-chain fatty acid ratios within microbial communities from the human colon, Appl Environ Microbiol, 71 (2005) 3692-3700.
[75] A. Barcenilla, S.E. Pryde, J.C. Martin, S.H. Duncan, C.S. Stewart, C. Henderson, H.J. Flint, Phylogenetic relationships of butyrate-producing bacteria from the human gut, Appl Environ Microbiol, 66 (2000) 1654-1661.
[76] A. Csordas, Butyrate, aspirin and colorectal cancer, European journal of cancer prevention : the official journal of the European Cancer Prevention Organisation (ECP), 5 (1996) 221-231.
[77] A. McIntyre, P.R. Gibson, G.P. Young, Butyrate production from dietary fibre and protection against large bowel cancer in a rat model, Gut, 34 (1993) 386-391.
[78] D.R. Janero, B. Burghardt, Thiobarbituric acid-reactive malondialdehyde formation during superoxide-dependent, iron-catalyzed lipid peroxidation: influence of peroxidation conditions, Lipids, 24 (1989) 125-131.

CHAPTER III

Development of a DNA adductomics methodology

Adapted from:

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ABSTRACT

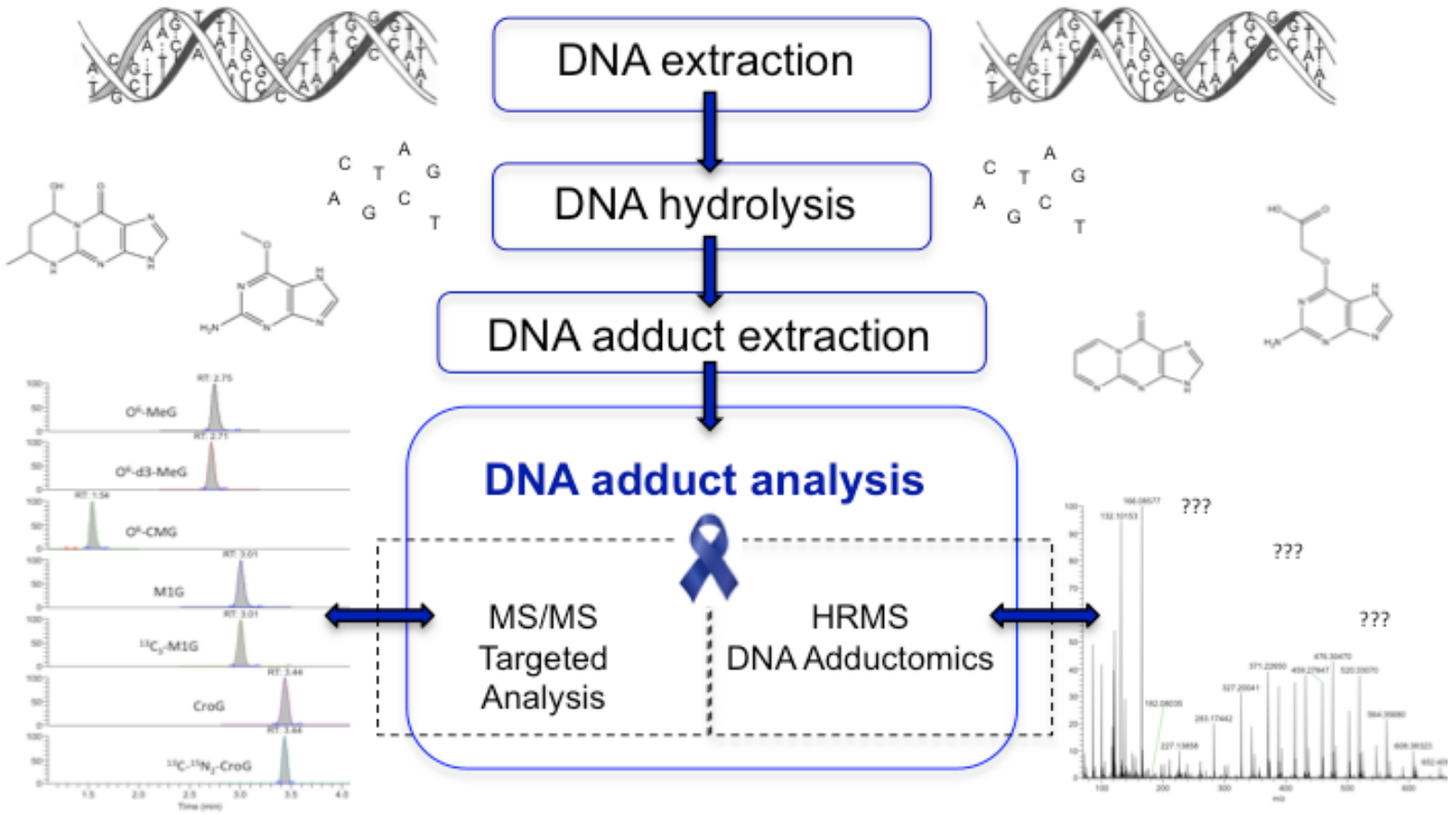
Exposure of DNA to endo- and exogenous DNA binding chemicals can result in the formation of DNA adducts and is believed to be the first step in chemically induced carcinogenesis. DNA adductomics is a relatively new field of research which studies the formation of known and unknown DNA adducts in DNA due to exposure to genotoxic chemicals. In this study, a new UHPLC-HRMS(/MS)-based DNA adduct detection method was developed and validated. Four targeted DNA adducts, which all have been linked to dietary genotoxicity, were included in the described method; O^6-MeG, O^6-CMG, pyrimido[1,2-a]purin-10(1H)-one (M_1G) and α-methyl-γ-hydroxy-1,N_2-propanoguanine (CroG). As a supplementary tool for DNA adductomics, a DNA adduct database, which currently contains 123 different diet-related DNA adducts, was constructed. By means of the newly developed method and database, all 4 targeted DNA adducts and 32 untargeted DNA adducts could be detected in different DNA samples. The obtained results clearly demonstrate the merit of the described method for both targeted and untargeted DNA adduct detection *in vitro* and *in vivo*, whilst the diet-related DNA adduct database can distinctly facilitate data interpretation.

Keywords:

Colorectal cancer, Dietary chemicals, Deoxyribonucleic acid adduct database,

High resolution mass spectrometry, Quadrupole-Orbitrap

GRAPHICAL ABSTRACT



1. INTRODUCTION

When a genotoxic chemical binds to DNA, chemically stable DNA adducts can be introduced to the DNA sequence. Carcinogenesis is known to arise due to a combination of both genetic and environmental factors, and DNA adduct formation is considered to be the first step in chemically induced carcinogenesis [1, 2]. Different 'endogenous' DNA adduct types are formed continuously due to normal physiological processes like oxidative stress and inflammation. The same and also other DNA adduct types can be formed due to exposure to environmental chemicals, which results in the occurrence of 'exogenous' DNA adduct levels [3]. It has been demonstrated that exogenous DNA adduct formation causally links the consumption of aflatoxin B1 contaminated food to hepatocellular carcinoma incidence [4] and more recently, researchers also established a causal link between the smoking of tobacco, the formation of PAH-DNA adducts and cervical cancer [5]. In consequence, research on DNA adduct related carcinogenesis due to certain environmental factors has gained an intelligible interest during the past decade.

Colon cancer incidence is mainly influenced by the presence (or absence) of particular environmental factors, as literature states that up to 70 % of colon cancer cases could be prevented by dietary changes [6, 7]. For example, in developed ('Western') countries, high red and processed meat consumption has been linked to a significantly higher colon cancer incidence in both men and women.

Different hypotheses have been put forward to substantiate this meat-cancer relationship. The two remaining hypotheses however, discuss the role of NOC and aldehyde formation in the human gut upon digestion of red and processed meat [8]. Both NOCs and aldehydes can bind to DNA nucleobases, resulting in the possible formation of a multitude of DNA adducts [6-9]. To study the role of meat consumption in particular, and the human diet and exosphere in general, in the initiation and progression of cancer, the development of a highly specific and sensitive method for the detection of diet-related DNA adducts could prove very useful. Different analytical methods such as immunoassays, immunohistochemistry, ^{32}P-postlabeling, GC- or HPLC-ECD, HPLC-FD, GC- or (HP)LC-MS, (LC-)NMR, LM-PCR and AMS [10, 11] have been developed for the detection of specific or bulky DNA adducts. Although every analytical method has its advantages, MS may prove to be the most useful analytical method for the screening, detection and quantification of diet-related DNA adducts [11, 12]. The most widely

used DNA adduct detection technique is ^{32}P-postlabeling, which has excellent sensitivity, but lacks adequate specificity [13]. Ultra-high performance liquid chromatography (UHPLC) combined with high resolution MS (HRMS) detection techniques on the other hand, enable accurate identification of analytes based on chemical composition and exact compound mass. Furthermore, MS techniques can also provide structural information and due to ongoing technical improvements, MS currently offers an excellent qualitative and quantitative tool for DNA adduct research [10, 14-16]. Although the advantages of HRMS are clear, it may lack sufficient sensitivity for quantification of low levels of DNA adducts. An alternative approach is the use of MS/MS, which most often brings about lower detection limits, enabling a more accurate quantitation. A recent advance in MS technology now combines the specificity of HRMS and the sensitivity of triple quadrupole MS/MS by combining both methodologies in 'hybrid quadrupole MS' systems [17, 18]. In other words, MS can easily measure low DNA adduct levels with the highest specificity, enabling simultaneous identification and quantification of different compounds.

In light of these advances, the possibilities of a hybrid HRMS/MS approach was investigated through the development and validation of a method capable of quantifying 4 DNA adducts that may be related to the meat and colon cancer hypothesis: O^6-MeG, O^6-CMG, M_1G and CroG. The formation of O^6-MeG and O^6-CMG has been linked to NOC formation in the gut during meat digestion [6], while M_1G and CroG can be produced when diet-related or endogenously formed MDA or crotonaldehyde (both LPOs) bind to guanine (G) in DNA [9]. Besides the described targeted approach (UHPLC-HRMS/MS), an untargeted full scan UHPLC-HRMS approach was explored to enable future DNA adductomics research. In addition, to facilitate DNA adduct research, known diet-related DNA adducts were defined and listed in an in-house database.

2. MATERIALS AND METHODS

2.1 Reagents and chemicals

Standards of M_1G, CrodG (α-methyl-γ-hydroxy-1,N_2-propano-2'-deoxyguanosine) and their internal standards M_1G-$^{13}C3$ and CrodG-^{13}C,$^{15}N_2$ were obtained from Toronto Research Chemicals (Toronto, Canada). O^6-CMdG was kindly provided by Dr. S. Moore (Liverpool John Moores University, UK), whilst O^6-MedG, O^6-d_3-MedG and a guanine (G) standard were purchased at Sigma-Aldrich (St. Louis, USA).

EtDA) (the precursor of KDA), crotonaldehyde (CRO) and 1,1,3,3-tetramethoxypropane (the precursor of MDA were obtained from Sigma-Aldrich (St. Louis, USA) as well.

2.2 Preparation of stock and working solutions

Prior to preparation of stock and working solutions, O^6-CMdG, O^6-MedG, O^6-d_3-MedG, CrodG and CrodG-^{13}C,$^{15}N_2$ were hydrolyzed from guanosines (nucleotides) to guanines (nucleobases) in 0.1 M formic acid at 80°C during 30 min [19]. All standards were diluted in MeOH and stored (-20°C) in stock and working solutions of respectively 500 ng/mL and 5 ng/ mL.

An 800 mM stock solution of KDA was synthesized through alkaline hydrolysis of EtDA [20]. After further dilution with phosphate buffered saline (PBS), a working solution of 20 mM KDA was obtained (Caution!: KDA is highly toxic and carcinogenic).

CRO was diluted to a stock and working solution of 800 and 20 mM PBS (Caution! CRO is highly toxic and carcinogenic). Stock and working solutions of both KDA and CRO were stored in dark glass bottles at -80°C.

The precursor of MDA; 1,1,3,3-tetramethoxypropanewas diluted in acidified (HCl, pH 2), deionized water and kept at 45°C during 1 h to ensure full release of MDA. Working solutions of MDA (20 mM) were freshly prepared prior to each experiment.

2.3 DNA hydrolysis and DNA adduct purification

DNA adducts were extracted and purified according to the protocol of Vanden Bussche et al. [19], during which DNA samples are initially subjected to DNA hydrolysis (30 min, 80°C) in 0.1 M formic acid to cleave both adducted and non-adducted DNA nucleobases from the DNA sequence. This is then followed by purification and sample cleanup by means of solid-phase extraction (SPE) (Oasis® HLB cartridges (1 cc, 30 mg) Waters (Milford, USA)). After SPE, the collected samples were evaporated to dryness under vacuum (90 min, 20°C) and resuspended in 100 mL of mobile phase. The obtained DNA adduct samples were stored at -20°C until LC-MS analysis.

2.4 UHPLC-HRMS(/MS) analysis

Chromatographic separation of DNA adducts was carried out by reversed phase chromatography. To ensure an optimal chromatographic separation and resolution, several column types were tested, including Nucleodur ISIS (5 mm, 3 x 150 mm and 4 x 250 mm) and Pyramid (1.8 mm, 2 x 100 mm) (Machery-Nagel, Düren, Germany), Hypersil Gold (1.9 mm, 2.1 x 100 mm, Thermo Fisher Scientific, San José, USA), Luna NH2 (5 mm, 4.60 150 mm, Phenomenex, Torrance, USA), Acquity BEH C18 (1.7 mm, 2.1 x 100 mm), HSS C18 (1.8 mm, 2.1 x 100 mm) and HSS T3 (1.8 mm, 2.1 x 100 mm, Waters, Milford, MA, USA). Different mobile phases containing different percentages of solvent modifiers including acetic or formic acid and ammonium bicarbonate, and solvents i.e. water, methanol and acetonitrile were tested in parallel, and were pumped at 300 mL per min by an Accela 1250 pump coupled to an Accela Autosampler (Thermo Scientific, San José, USA).

MS analysis was performed on a hybrid Quadrupole-Orbitrap High Resolution Accurate Mass Spectrometer (HRAM, Q-Exactive™, Thermo Fisher Scientific, San José, USA) coupled to a heated elektrospray ionization (HESI-II) source. All HESI and HRMS(/MS) parameters were optimized for the 4 targeted compounds and their internal standards. Chemical composition of the studied DNA adducts, theoretical and measured masses, observed mass deviation, monitored MS/MS ions and expected retention time (RT) are shown in table 1.

General instrument control and data processing were performed with Xcalibur™ 3.0 and ToxID™ software (Thermo Fisher Scientific, San José, USA).

Table 1. Chemical composition, theoretical mass, measured mass, observed mass deviation, monitored MS/MS product ions, optimal collision energy and expected RT of the studied DNA adducts.

Name	Chemical formula	Theoretical mass (H^+)	Measured mass (H^+)	Mass deviation (ppm)	Monitored MS/MS product ion (H^+)	MS/MS collision energy (NCE)	RT (min)
O^6-CMG	$C_7H_7N_5O_3$	210.06217	210.06194	1.09	152.05663	40	1.5
O^6-MeG	$C_6H_7N_5O$	166.07234	166.07225	0.54	134.04613	60	2.8
d_3-MeG	$C_6H_4[^2H]_3N_5O$	169.09117	169.09100	1.01	134.04617	60	2.7
M_1G	$C_8H_5N_5O$	188.05669	188.05649	1.06	97.04008	60	3.0
$^{13}C_3$-M1G	$C_5[^{13}C]_3H_5N_5O$	191.06675	191.06655	1.05	100.05006	60	3.0
CroG	$C_9H_{11}N_5O_2$	222.09855	222.09843	0.54	152.05670	40	3.5
$^{13}C^{15}N_2$-CroG	$C_8[^{13}C][^{15}N]_2H_{11}N_3O_2$	225.09598	225.09560	1.69	155.05392	40	3.5

2.5 Validation in calf thymus DNA

Currently, guidelines or regulations for the validation of analytical methods for the detection of DNA adducts in biological matrices are not available. The US Food and Drug Administration (FDA) did announce its intent to publish guidelines for biomarker detection methods, but a final draft has not yet been published [21]. Therefore, the guidelines for chemical methods described by the US FDA [22] and the EU Commission Directive 2002/657/EC [23] on the performance of analytical methods were taken into account. The described analytical method was validated by assessment of specificity, selectivity, linearity, precision and trueness with full scan HRMS. Quantification and detection limits were assessed with and for the full scan HRMS, SIM-HRMS and SIM-HRMS/MS method. The matrix in which all validation parameters were tested, consisted of the commercially available Calf Thymus DNA (CT-DNA). Lyophilized CT-DNA was purchased from Rockland (Gilbertsville, Pennsylvania, USA) and stored at 4°C in Tris-EDTA buffer (1 mg/mL).

2.6 DNA adducts in CT-DNA treated with genotoxic compounds

The genotoxic compounds KDA, MDA and CRO were supplemented to CT-DNA in triplicate and incubated overnight (37°C) to allow interaction and subsequent DNA adduct formation. KDA was added to 100 mg of DNA in 3 different concentrations (1 mM, 2.5 mM and 5 mM). Separately, both MDA and CRO were added to 100 mg of DNA as well, but in concentrations of 0.1 mM, 0.25 mM and 0.5 mM. After overnight incubation, DNA adducts were extracted and analyzed. An equal amount of CT-DNA (100 mg), not treated with genotoxic compounds, was used as a negative control and to correct for intrinsically present DNA adduct levels.

2.7 DNA adduct profiling in colon biopsies

Left colon tumors were obtained during colonoscopy of 10 individuals diagnosed with colon cancer at the Ghent University Hospital. The average age of the patients was 74, the youngest being 58 and the eldest 83. The group consisted of 7 men and 3 women. All resected samples were poorly to moderately differentiated primary adenocarcinomas which were submerged and stored in 95 % of ethanol at -80°C. Two samples were retrieved in 2006 and 2007, but most were gathered in 2012 and 2013.

A Qiagen blood & tissue kit (Qiagen, Hilden, Germany) was used for the extraction of DNA from the tissue samples according to the protocol provided by the manufacturer. The concentration and purity of the extracted DNA was determined with a Nanodrop ND-1000 spectrophotometer (Isogen Lifescience, Ijsselstein, The Netherlands). Following this, the obtained DNA in each sample was hydrolyzed and DNA adducts were purified according to the protocol described under 2.3 DNA hydrolysis and DNA adduct purification.

3. RESULTS

3.1 UHPLC-HRMS(/MS) settings optimization

The Acquity BEH C18 Waters column and the use of 0.05 % of acetic acid in water and 100 % methanol as mobile phases provided optimal results for baseline peak separation, signal-to-noise ratio, peak area, peak shape and RT of all eluting targeted DNA adducts. The amount of methanol was increased linearly from 0.85 min, reaching 50 % after 4 min, which was then immediately followed by 1 min of 100 % methanol. For the remaining 2 min, the column was re-equilibrated at 95:5 0.05 % acetic acid in water:methanol.

Three different MS acquisition modes were assessed: full scan MS, SIM-MS (selected ion monitoring) and SIM-MS/MS. Both negative and positive ionization mode (polarity switching) were included to not only enable the targeted analysis of the 4 targeted DNA adducts, but also the future analysis of all untargeted DNA adducts of interest, thus facilitating the use of this method as an "omics" tool. Likewise, the full MS scan range was held at 70-700 m/z to enable an untargeted analysis next to the targeted analysis. A scan range of 100-250 m/z was chosen for the targeted analysis in SIM-MS and SIM-MS/MS. Ultimately, the chosen scan resolution for full MS was 'ultra high' (100,000 FWHM (Full Width Half Maximum)), using 3 microscans. Optimal maximum inject time was 500 ms and the automatic gain control target was best operated in 'high dynamic range' (3 x e^6). Sheath gas, auxiliary gas and sweep gas flow rate were set at 35, 5 and 2 arbitrary units. Optimal spray voltage, capillary temperature, capillary voltage and heater temperature were kept at 4 kV, 280°C, 40 V (positive or negative, dependent on polarity switch) and 330°C respectively. S-lens RF-level was set at 90 and the same settings were applied for all acquisition types. However, a resolution of 70,000 and 17,500 FWHM appeared sufficient for

respectively SIM-MS and SIM-MS/MS, whilst a maximum inject time of 250 ms was applied. Normalized collision energy (NCE) and monitored product ions in MS/MS for each targeted DNA adduct and their internal standards are shown in table 1. A chromatogram and full scan mass spectrum of all targeted compounds and their internal standards are presented in figure 1 and figure 2, respectively.

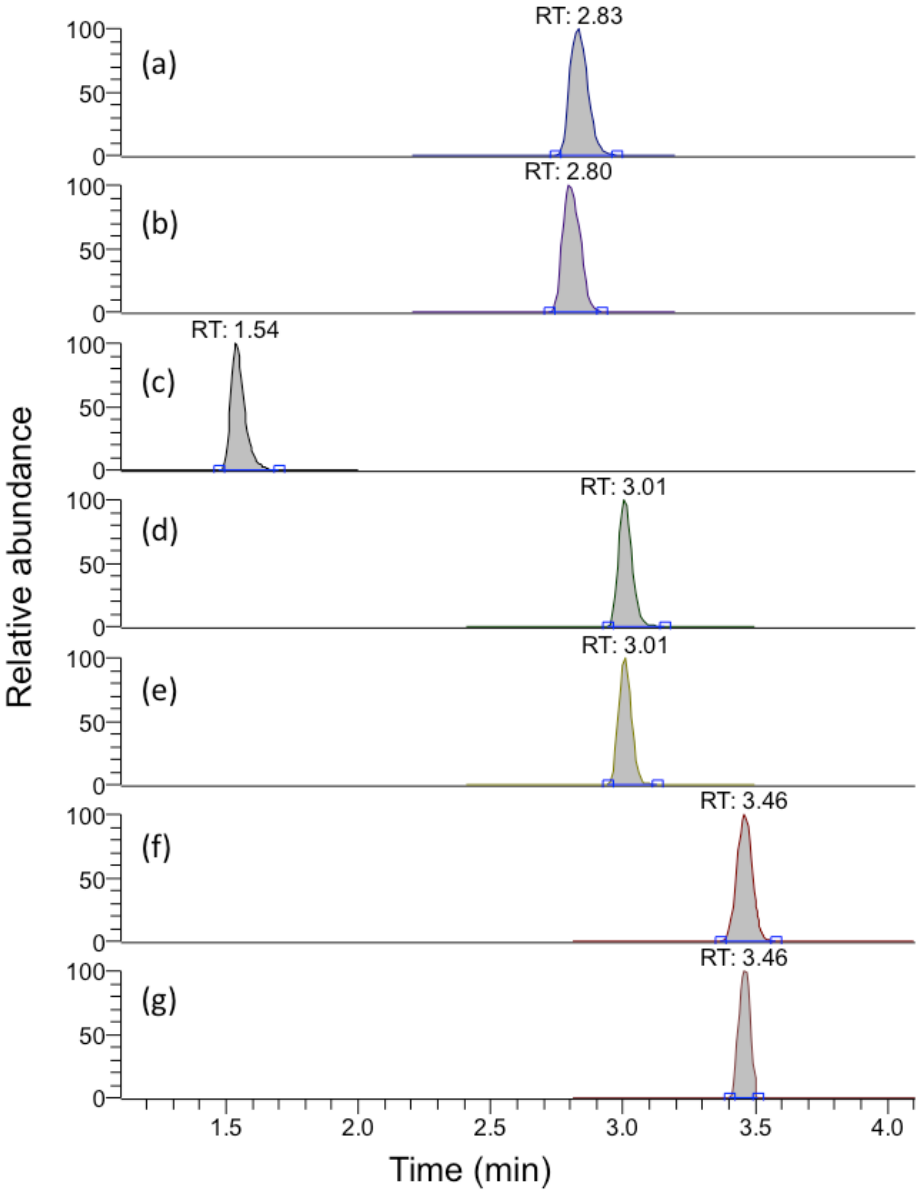


Figure 1. Chromatogram of O^6-MeG (a), O^6-d_3-MeG (b), O^6-CMG (c), M_1G (d), $^{13}C_3$-M_1G (e), CroG (f) and $^{13}C^{15}N_2$-CroG (g) in CT-DNA with SIM-MS/MS acquisition.

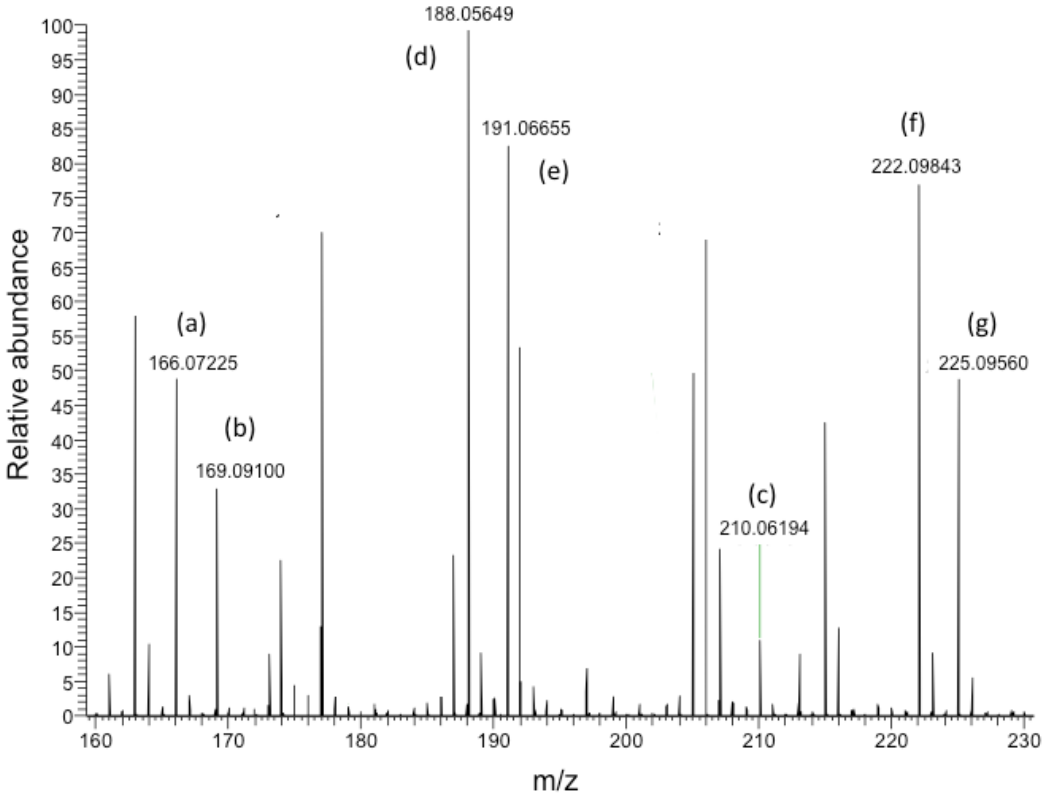


Figure 2. Mass spectrum of O^6-MeG (a), d_3-MeG (b), O^6-CMG (c), M_1G (d), $^{13}C_3$-M_1G (e), CroG (f) and $^{13}C^{15}N_2$-CroG (g) in CT-DNA with HRMS acquisition.

3.2 Validation of the full HRMS method for the targeted detection of DNA adducts

3.2.1 Specificity

The specificity of the HRMS method for the detection of O^6-CMG, O^6-MeG and CroG was assessed by analyzing over 21 blank CT-DNA samples and over 108 spiked CT-DNA samples. Spiked samples were fortified with a mixture of all DNA adducts, or each DNA adduct separately at three different concentrations levels (2.50, 5.00 and 7.50 ng/mL for O^6-CMG and O^6-MeG; 0.25, 0.50 and 0.75 ng/mL for CroG). Comparison of the obtained chromatograms of blank and spiked DNA samples demonstrated that the DNA matrix did not cause interference for O^6-CMG, O^6-MeG and CroG detection. In addition, spiking of O^6-CMG, O^6-MeG and CroG lead to a significant increase in peak intensity with a signal-to-noise ratio > 3. For the M_1G DNA adduct, specificity could not be thoroughly assessed by comparison of blank and fortified

samples, since the matrix DNA contained endogenous levels of M_1G. However, no other matrix substances significantly interfered with the analysis of M_1G (signal-to-noise ratio > 3) and spiking of three different levels of M_1G (0.25, 0.50 and 0.75 ng/mL) to CT-DNA did significantly increase the M_1G peak area at the expected RT.

3.2.2 Selectivity

The methods' selectivity could be demonstrated by analyte identification based on relative RT (ratio of analyte RT and corresponding internal standard RT) and the accurate mass of their positive ions ($[M+H^{+}]$.). Only chromatographic peaks of interest with signal-to-noise ratios that exceeded 3 were taken into account and tolerance levels for relative RT and maximum mass deviation were established at 2.5 % and 5 ppm respectively.

3.2.3 Linearity

Linearity was assessed by deploying 2 separate calibration curves in triplicate. A first calibration curve was established in a low range that would enable quantification of endogenous and exogenous levels of DNA adducts [3, 24][3,24], whereas the second calibration curve was used to enable the *in vitro* application of this method (see 2.6 DNA adducts in CT-DNA treated with genotoxic compounds). For the calibration curves, the matrix DNA was fortified with 0.025; 0.05; 0.075; 0.1; 0.125; 0.25; 0.50; 0.75; 1; 1.25; 2.5; 5; 7.5; 10 and 12.5 ng/mL O^6-CMG, O^6-MeG, M_1G and CroG. The obtained coefficients of determination (R^2) proved to be over 0.99, suggesting a good to excellent linearity, which could be confirmed by means of a one-way ANOVA (F-test) linearity test ($p > 0.05$). The ANOVA model was constructed with the detected area ratio of each component (DNA adduct peak area/area of the internal standard) as the dependent variable and the calibration curve concentration levels as the independent variable (SPSS Statistics 21).

3.2.4 Mean recovery

To assess mean recovery of the targeted compounds, 3 series of six replicates of the earlier described CT-DNA samples fortified with three different spike levels were utilized. As M_1G

appeared to be endogenously present in CT-DNA, total concentrations were adjusted to the measured fortified concentrations prior to calculation of the corrected mean recovery. Mean recovery of M_1G, CroG and O^6-MeG were within the narrow range of 97 and 104 % and thus proved to be excellent. O^6-CMG mean recoveries ranged between 89 and 108 %, which is still well within the acceptable range of 80-120 %.

3.2.5 Precision

To evaluate precision, repeatability and intra-lab reproducibility were determined by calculation of the relative standard deviations (RSD %). Three sets of six fortified samples (equals the three fortification levels with six replicates each) were analyzed to test repeatability. One additional set of six samples fortified, extracted and analyzed by a different person at a different time enabled assessment of intra-lab reproducibility. As no detectable O^6-CMG, O^6-MeG and CroG levels appeared to be present in the matrix, and since endogenous levels in different tissues demonstrate a certain variation, fortification levels for O^6-CMG were selected according to the estimated limit of detection and quantification with the full HRMS method. To establish M_1G levels, the endogenous levels of M_1G in the matrix DNA were taken into account. For CroG and O^6-MeG, spike levels were adjusted to those of M_1G and O^6-CMG respectively, due to their analogue nature and origin. Also, for M_1G, the endogenously present M_1G DNA adduct levels in the CT-DNA were subtracted from the total measured M_1G concentrations in spiked CT-DNA prior to calculation of the final/fortified concentration and RSD %. For M_1G, CroG and O^6-MeG, repeatability appeared to be excellent since RSD % consistently proved below 4 % for each analyte and fortification level. In comparison, repeatability for O^6-CMG was less good, but still below the acceptable RSD % limit of 15 with a RSD % of 9. Intra-lab reproducibility for all targeted compounds is good as well, with all RSD % below 9.

3.2.6 Limits of detection and quantification

LOQs were determined at a minimal signal-to-noise ratio of 10, whereas limits of detection (LODs) required a signal-to-noise ratio of at least 3 (table 2). LODs and LOQs with SIM-MS and SIM-MS/MS clearly exceeded the full HRMS LODs and LOQs for O^6-CMG and O^6-MeG. For CroG and M_1G, differences in LODs and LOQs between full HRMS, SIM-MS and SIM-

MS/MS were less pronounced. Practical assessment of LOD and LOQs for M_1G is limited by the presence of M_1G levels inherent to the CT-DNA matrix. Therefore, average LOD and LOQs levels for M_1G were assessed based on co-chromatography of added M_1G with endogenously present M_1G in the CT-DNA matrix. For the combined detection and quantification of all 4 targeted DNA adducts, SIM-MS/MS would be preferred over SIM-MS and full MS since it combines the lowest LOQs for all 4 targeted compounds.

Table 2. Obtained validation parameters for each targeted DNA adduct including mean recovery, repeatability, intra-lab reproducibility, LODs and LOQs.

Analyte	Spike levels (ng per 100 µg DNA)	Mean Recovery ± SD (%)	Repeatability RSD ± SD (%)	Intra-lab reproducibility RSD (%)	LOD full MS (adducts per 10^8 nucleotides)	LOQ SIM (adducts per 10^8 nucleotides)	LOQ MS/MS (adducts per 10^8 nucleotides)
O^6-CMG	1.25	89.5 ± 9.0	8.4 ± 4.8	5.6	888	22.2	22.2
	2.50	107.9 ± 4.4	7.0 ± 2.9	5.8			
	3.75	106.6 ± 3.1	7.1 ± 2.9	8.3			
O^6-MeG	1.25	103.8 ± 0.8	1.7 ± 0.2	3.6	28.1	28.1	2.82
	2.50	100.2 ± 1.8	2.5 ± 0.4	1.8			
	3.75	101.6 ± 1.5	1.5 ± 0.6	2.1			
M_1G	0.125	103.3 ± 0.3	3.7 ± 1.5	4.4	2.48	2.48	4.96
	0.250	97.3 ± 1.9	2.5 ± 0.5	6.9			
	0.375	100.2 ± 0.5	2.1 ± 0.7	3.8			
CroG	0.125	97.4 ± 1.4	2.2 ± 0.8	5.8	0.52	1.05	4.20
	0.250	97.0 ± 1.3	2.8 ± 0.9	3.9			
	0.375	98.0 ± 2.0	1.8 ± 1.0	3.6			

3.3 Development of a diet-related DNA adduct database to facilitate interpretation of untargeted DNA adductomics studies

To construct an in-house database, literature was searched for DNA adducts of which harmful exogenous levels may be linked to the human diet. The main focus of the search were DNA adduct types originating from DNA alkylation or oxidation since these reaction types are of particular interest regarding the mechanisms behind colon cancer initiation and promotion by red and processed meat consumption [8, 25]. To this purpose, ISI Web of Knowledge and PubMed databases were searched for relevant papers using combinations of the grouped search terms (Food* OR Diet*) AND ('DNA adduct' OR 'DNA damage'). Upon retrieval of papers documenting DNA adducts inducing foods, chemicals and/or metabolites, search terms were specified; e.g. 'Meat' AND 'Heterocyclic amines' AND 'DNA adduct' to retrieve all relevant papers and information on the topic at hand. The hence retrieved DNA adducts were assembled in a database containing 123 different diet-related DNA adducts (table 3). By means of ToxID™ software (Thermo Fisher Scientific, San José, USA) and the self-constructed database, the full scan HRMS spectra of different DNA samples could be screened for the presence of non-targeted diet-related DNA adducts in retrospect. The considered inclusion criteria consisted of a minimal signal intensity of 10,000, a maximum mass deviation of 10 ppm, recurrence and stable RT of the DNA adduct of interest in replicate and repeat samples and a C^{12}/C^{13} ratio approaching the natural 99:1 ratio. Detection of a certain diet-related DNA adduct with full MS and Tox-ID™ profiling renders chromatograms and data on measured peak area of the masses of interest and can thus suggest the presence of a putative DNA adduct in a sample. If believed relevant, exact confirmation of DNA adduct identity can be made by use of commercially available analyte standards. In this study, normalization of the obtained untargeted DNA adduct Tox-ID™ data was based on the measured signal intensity (area) of the guanine nucleobase in each sample by expressing DNA adduct area relative to guanine area. The identity of guanine was confirmed with an analytical standard. To demonstrate the possible merit and application of the described approach, full HRMS spectra obtained from blank CT-DNA, CT-DNA treated with KDA, MDA and CRO, and also tumor biopsies were interpreted by means of ToxID™ profiling and the self-constructed database. The results are discussed in the related sections below and documented in figure 3.

Table 3. Diet-related DNA adducts included in the DNA adduct database (anno 2015[*]).

Origin/type of DNA adduct	Guanine (G) adducts	Adenine (A) adducts	Cytosine (C) adducts	Thymine (T) adducts
Alkylation [3, 26-41]	Butyl-G	Butyl-A	Butyl-C	Butyl-T
	Carboxyethyl-G[a]	Carboxyethyl-A[a]	Carboxyethyl-C[a]	Carboxyethyl-T
	Carboxy-G	Carboxyl-A	Carboxyl-C	Carboxy-T
	Carboxymethyl-G	Carboxymethyl-A	Carboxymethyl-C	Carboxymethyl-T
	Dimethyl-G	Dimethyl-A	Dimethyl-C	Dimethyl-T
	Ethyl-G[b]	Ethyl-A[c]	Ethyl-C	Ethyl-T
	Hydroxyethyl-G	Hydroxyethyl-A	Hydroxyethyl-C	Hydroxyethyl-T
	Hydroxymethyl-G	Hydroxymethyl-A	Hydroxymethyl-C	Hydroxymethyl-T
	Methoxymethyl-G	Methoxymethyl-A	Methoxymethyl-C	Methoxymethyl-T
	Methyl-G	Methyl-A[c]	Methyl-C	Methyl-T
	Nitro-G	Nitro-A	Nitro-C	Nitro-T
	Propyl-G	Propyl-A	Propyl-C	Propyl-T
	Tetramethyl-G	Tetramethyl-A	Tetramethyl-C	Tetramethyl-T
	Trimethyl-G	Trimethyl-A	Trimethyl-C	Trimethyl-T
(Lipid per-)oxidation [3, 35, 37, 42-51]	1,N2-etheno-G	1,N6-etheno-A	Alloxan	Formyl-U
	1,N2-propano-G[b]	Fapy-A	C-glycol	Hydroxy-T
	Fapy-G	Heptenal-etheno-A	Dihydroxy-C	Hydroxymethylhydantion
	Glyoxal-G	Hydroxy-A	Heptenal-etheno-C	Hydroxymethyl-U
	Heptenal-etheno-G	Hydroxynonenal-A	Hydroxy-C	T-glycol
	Heptenal-G	Malondialdehyde-A	Hydroxyhydantion	
	Hydroxynonenal-G	Malondialdehyde-3-A	Hydroxynonenal-C	
	Hydroxy-1,N2-propano-G	Oxohexenal-A	Hydroxy-U	
	Hydroxy-G	3,N4-etheno-A	Malondialdehyde-C	
	Imidazolone		Malondialdehyde-3-C	
	Malondialdehyde-G		Methyl-C-glycol	

Table 3 continued (1).

Origin/type of DNA adduct	Guanine (G) adducts	Adenine (A) adducts	Cytosine (C) adducts	Thymine (T) adducts
(Lipid per-)oxidation [3, 35, 37, 42-51]	Malondialdehyde-2-G Methylglyoxal-G N2,3-etheno-G Octanal-G Oxohexenal-G Oxazolone Pentenal-G		Oxohexenal-C Oxohexenal-methyl-C U-glycol	
Heterocyclic Amines [52-55]	AaC-G DiMeIQx-G Hydroxy-PhIP-G IQ-G IQx-G MeAaC-G MeIQ-G MeIQx-G PhIP-G			
Polycyclic Aromatic Hydrocarbons [56, 57]	Benzo(a)pyrene-G			
Mycotoxins [58, 59]	Aflatoxin-B1-G Aflatoxin-B1-Fapy-G Aflatoxin-M1-G Ochratoxin-G Sterigmatocystin-G			

Table 3 continued (2).

Origin/type of DNA adduct	Guanine (G) adducts	Adenine (A) adducts	Cytosine (C) adducts	Thymine (T) adducts
Acrylamide [60-62]	Carbamoylethyl-G Carbamoylhydroxy-ethyl-G	Carboxyhydroxyethyl-A Carbamoylhydroxy-ethyl-A		

[a]: also related to acrylamide exposure

[b]: also related to alcohol consumption

[c]: also related to tobacco smoke exposure

[*]: this table contains all diet-related DNA adduct types that were included in the in-house DNA adduct database in 2015; i.e. at the time when the in this chapter reported experiments were performed. There is a significant overlap with the DNA adducts listed in chapter I, which contains all diet-related DNA adducts that are currently (anno 2017) listed in the in-house DNA adduct database; the database is updated continuously.

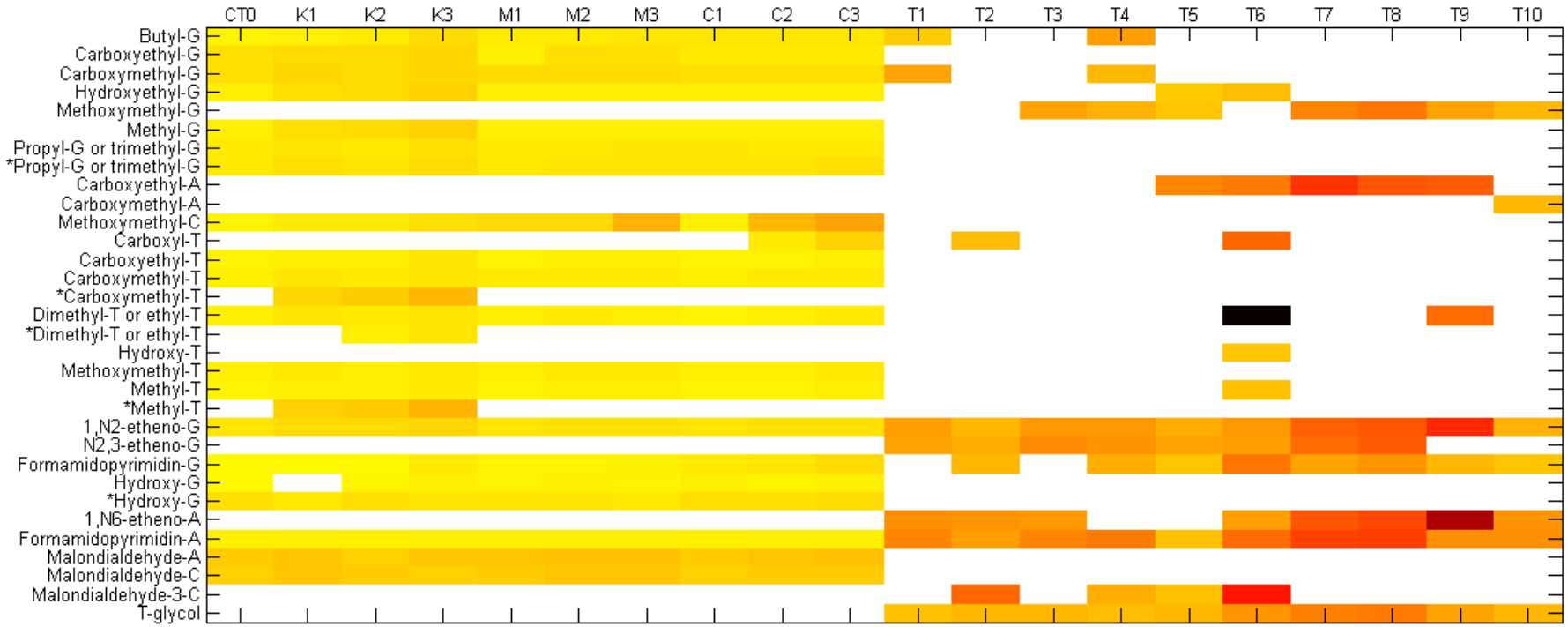


Figure 3. Heat map of DNA adduct profiling in CT-DNA and colon tumors: white to yellow and red to black represent low to high DNA adduct signal intensity (DNA adduct area to guanine area ratio), respectively. Blank CT-DNA data are presented on the left (CT0) and K1-K3, M1-M3 and C1-C3 show the effect of rising concentrations of respectively KDA (potassium diazoacetate), MDA and CRO on DNA adduct profile. Individual biopsy data are presented on the right (tumor (T) 1 to 10).

3.4 CT-DNA adducts

The commercially available CT-DNA contained endogenous levels of M1G, whereas no detectable endogenous levels of O^6-MeG, O^6-CMG and CroG could be detected. Next to the targeted cyclic M_1G DNA adduct, Tox-ID™ profiling indicated the possible presence of 20 additional DNA adducts (figure 3). This includes M_1A (oxopropenyl-A), M_1C (oxopropenyl-C), $1,N^2$-etheno-G, non-O^6-carboxymethyl-G, two non-O^6-methyl-Gs, carboxyethyl-G, Fapy-G (formamidopyrimidine-G), fapy-A (formamidopyrimidine-A), methyl-T, dimethyl-T or ethyl-T, carboxymethyl-T, carboxyethyl-T, methoxymethyl-C, methoxymethyl-T, butyl-G, hydroxyethyl-G, propyl-G, trimethyl-G and hydroxyl-G DNA adducts (figure 3).

3.5 DNA adducts in CT-DNA treated with genotoxic compounds

Upon addition of different concentrations of KDA, MDA and CRO to CT-DNA, O^6-MeG, O^6-CMG, M_1G and CroG DNA adducts were formed during overnight incubation. O^6-CMG and O^6-MeG were formed upon addition of KDA, CroG was formed due to addition of CRO and M_1G was produced after the addition of MDA; a clear dose-response effect was observed (figure 4). Based on the fact that LPOs are highly reactive chemicals, and also based on practical assessment of the experimental setup, MDA and CRO concentration levels were adjusted to avoid overload of the LC and MS system. Therefore, the added MDA and CRO concentrations are lower than the added KDA concentrations. Addition of KDA to DNA also resulted in the formation of untargeted DNA adducts (figure 3). A first observation was the possible presence of 2 additional methyl-G compounds other than O^6-MeG at RTs of 1.7 and 2.3 min. Both additional MeG DNA adducts were more prevalent than O^6-MeG. In addition to MeG, a slight increase in butyl-G, methoxymethyl-C and a significant increase in methyl-T and carboxymethyl-T could be noted after KDA treatment. During screening of CT-DNA treated with MDA by means of the DNA adduct database, it could be observed that not only M_1G, but also M_1A and M_1C levels appeared to increase in a dose-response manner when the MDA concentration was raised (figure 3). In addition, just like KDA, MDA conceivably promoted the formation of methoxymethyl-C. Untargeted analysis of CT-DNA samples that had been treated with CRO revealed the probable presence of methoxymethyl-C, fapy-G, hydroxy-G and carboxy-T.

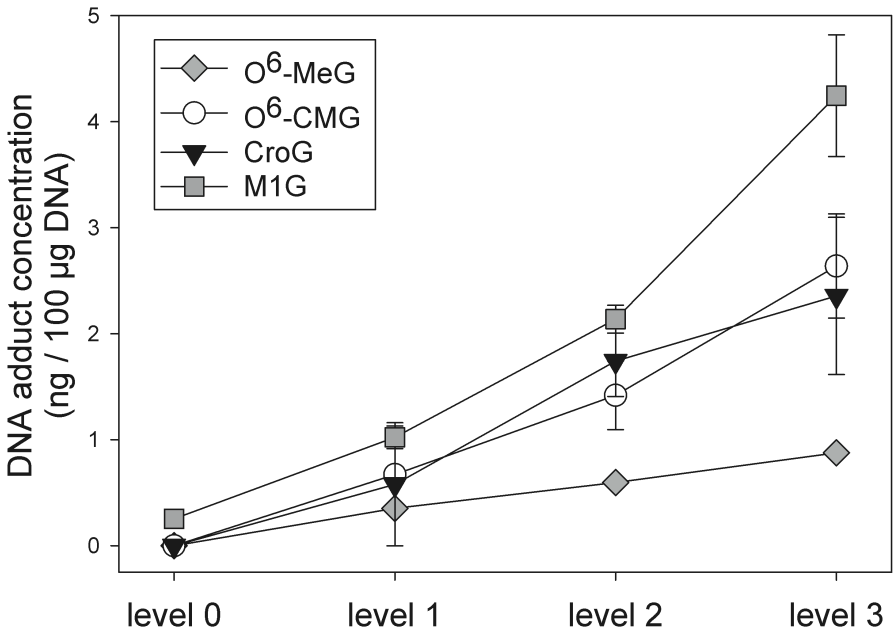


Figure 4. Mean (± s.d.) O^6-MeG, O^6-CMG, CroG and M_1G DNA adduct formation in CT-DNA after exposure to genotoxic chemicals. Level 0 corresponds to the negative control samples. Level 1 represents addition of 1 mM of KDA or 0.1 mM of CRO or MDA. Level 2 equals addition of 2.5 mM of KDA or 0.25 mM of CRO or MDA. Level 3 demonstrates addition of 5 mM KDA or 0.5 mM of CRO or MDA.

3.6 DNA adducts in colon biopsies

O^6-CMG could be identified and quantified in 8 out of 10 tumor samples. For quantification of O^6-CMG in each sample, the DNA adduct concentrations were adjusted according to the measured DNA concentration in each sample. The mean amount of O^6-CMG equaled 815 adducts per 10^8 nucleotides, with a relatively high inter-individual variability (range from < LOD to 1630 adducts per 10^8 nucleotides). M_1G, CroG and O^6-MeG could not be detected. Untargeted analysis of all tumor samples (T1-T10) was carried out in parallel. The list of observed DNA adducts includes both alkylation and oxidation DNA adducts, i.e. non-O^6-CMG (same mass as O^6-CMG, but a different RT), butyl-G, hydroxyethyl-G, carboxyethyl-A, carboxymethyl-A, methyl-T, dimethyl-T or ethyl-T, carboxy-T, fapy-G, fapy-A, T-glycol, hydroxyl-T, 1,N_2-etheno-G, N_2,3-etheno-G, 1,N_6-etheno-A, M_3C and methoxymethyl-G (figure 3).

4. DISCUSSION

A new UHPLC-HRMS/MS method was developed to enable targeted and untargeted detection of both known and unknown DNA adducts. A first focus was the separation and targeted detection of O^6-CMG, O^6-MeG, M_1G and CroG. These DNA adducts are of particular interest because DNA-damaging NOCs and LPOs appear to be the two main culprits in the complex underlying mechanism that links red and processed meat consumption to an elevated colon cancer risk [8, 25]. The combined targeted detection of O^6-CMG, O^6-MeG, M_1G and CroG is unique as it permits simultaneous investigation of 2 important but very different pathways. Furthermore, at the time, this is the only described MS-based method to combine the detection of O^6-CMG, O^6-MeG, M_1G and CroG.

DNA adductomics is an up-and-coming approach to investigate DNA adduct formation and its possible link to chronic disease. To elucidate supplementary meat or colon cancer related DNA adduct types and pathways, the use of this UHPLC-HRMS method was investigated i*n vitro* and *in vivo* ensuring valid future clinical application in DNA adductomic studies. To the best of our knowledge, this paper describes 1 of only 3 high resolution MS-based DNA adductome mapping methods, whereas it is the first to explore and illustrate the use of Quadrupole-Orbitrap technology for DNA adduct profiling purposes.

Supplementary, an in-house database that lists known DNA adducts, was constructed and implemented to facilitate the complex process of omics data interpretation.

4.1 DNA adduct analysis with UHPLC-HRMS/MS

All relevant performance characteristics of the MS method were in accordance with internationally accepted analytical criteria. However, because of the method's intended use in a clinical setting, most attention was paid to the achievable LODs and LOQs for the 4 targeted DNA adducts prior to *in vitro* and/or *in vivo* SIM-HRMS/MS method application.

The *in vivo* relevance of the detection of O^6-CMG has been demonstrated by its qualitative detection in several biological samples like blood, colon biopsies and exfoliated colonocytes [63-65]. To the best of our knowledge, average endogenous levels of O^6-CMG in human tissue have not yet been reported. Therefore, at present, a scientifically based statement on the applicability

of this method for the quantification of possibly low endogenous O^6-CMG DNA adduct levels is not possible. Cupid et al. described the measurement of 35-80 O^6-CMG DNA adducts per 10^8 nucleotides in blood of volunteers consuming a high meat diet [65]. These DNA adducts levels were detected by means of Immunoslot Blot with an LOD of 15 adducts per 10^8 nucleotides, which confirms the competitiveness of our targeted SIM-HRMS/MS method.

A correct quantification of endogenous levels of the O^6-MeG DNA adduct with an LOQ of 2.82 adducts per 10^8 nucleotides with MS/MS acquisition will depend on tissue or cell type. For example, Kang et al. reported the detection of less than one O^6-MeG DNA adduct in 10^8 nucleotides in leucocyte DNA, whereas more than two O^6-MeG adducts per 10^8 nucleotides appeared to be present in hepatic DNA [3, 41]. This suggests that our current method allows detection and quantification of O^6-MeG in a clinically relevant range. If required, the current LOQ and LOD could be improved by tweaking the MS and HESI settings. However, at the time, all settings were optimized for the simultaneous detection of all 4 targeted DNA adducts and for O^6-CMG, demonstrating the highest LOQ and LOD, in particular.

The retrieved LOQs for M_1G and CroG (<5 adducts per 10^8 nucleotides) appeared to be more than sufficient. Indeed, according to previously reported endogenous levels of M_1G and CroG in several tissue types, all reported DNA adduct levels exceeded 6 adducts per 10^8 nucleotides [3], which implies that our LOQs are below endogenously present M_1G and CroG DNA adduct levels.

4.2 *In vitro* application

The presence of both targeted and untargeted DNA adducts were investigated in blank CT-DNA or CT-DNA treated with genotoxic chemicals to demonstrate the *in vitro* application of the described method and the in-house constructed database.

In blank CT-DNA samples, different DNA adducts could be retrieved. M_1G appeared to be the sole detected targeted DNA adduct endogenously present in CT-DNA and similar amounts of M_1G in CT-DNA have been reported before [66]. Using the untargeted HRMS approach, the accurate masses of 20 additional compounds (figure 3) could be linked to DNA adducts included in the self-constructed diet-related DNA adduct database (see table 3), resulting in a putative identification. The endogenous or exogenous occurrence of several of these DNA adducts in

DNA and their link to for example oxidative stress [35, 50], exposure to environmental carcinogens [40, 67] and carcinogenesis [50, 68], have been reported previously, acknowledging their relevance.

Treatment of CT-DNA with KDA, MDA and CRO resulted in the formation and detection of the 4 targeted DNA adducts. The demonstrated dose response effect was to be expected for KDA, a well-known NOC, and both O^6-MeG and O^6-CMG, since the methylation and carboxymethylation of deoxyguanosine at the O^6 position by nitrosated glycine derivatives have been documented in the past [19, 69]. The demonstrated formation of M_1G in CT-DNA by attack of the G base by the highly reactive LPO MDA has also been reported extensively in literature, with M_1G being the predominantly formed DNA adduct [43]. The same applies for CRO and CroG formation [9]. The more 'efficient' and pronounced interaction of MDA and CRO with DNA to form M_1G and CroG, compared to the interaction of KDA with DNA to form O^6-CMG and O^6-MeG (lower added MDA and CRO concentrations (compared to KDA) yield similar DNA adduct concentrations; figure 4) can be explained by the highly reactive nature of LPOs [35].

Next to the detection of targeted DNA adducts, application of the HRMS method also enabled the detection of untargeted DNA adducts. Rapid putative identification of these compounds in retrospect was empowered by the use of the self-constructed database. In total, 25 untargeted DNA adducts were detected in blank CT-DNA and/or upon treatment with KDA, MDA and/or CRO. Several of these putatively identified DNA adducts have been detected previously and described in literature (table 3), acknowledging their relevance. Retrieval of these DNA adducts clearly emphasizes the potential of the HRMS method for DNA adductome mapping and the ease of putative DNA adduct identification by means of our DNA adduct database.

Some of the untargeted DNA adducts reported above have not been investigated extensively. However, considering the possible detection of all these compounds after incubation of DNA with KDA, MDA or CRO, further investigation is warranted. After all, extensive research on the possible link between toxic endo- and exogenous NOCs, LPOs and disease has been conducted in the past and has revealed a connection to an increased cancer risk [70, 71].

4.3 *In vivo* application

The described DNA adductomics method has been constructed for future use in clinical studies. Therefore, preliminary tests for *in vivo* application were conducted on 10 colon tumor tissue samples. This resulted in the detection of the O^6-CMG DNA adduct in a vast majority of colon tumor biopsies. The other 3 targeted compounds could not be retrieved in any of the biopsy samples, although they have been detected in different human tissue types before [3], thus suggesting that M_1G, CroG and O^6-MeG were either absent or no longer present above the LOD in the analyzed biopsies. ToxID™ screening revealed the likely presence of 17 untargeted DNA adducts of which some could be retrieved in all 10 biopsies, whilst others could only be detected in some or just one sample(s).

Current knowledge on the stability of different DNA adducts is still limited. In consequence, future DNA adduct profiling should be carried out as soon as possible following tissue sampling to avoid DNA adduct instability issues. As these possible stability issues were not sufficiently taken into account during colon biopsy sample handling and storage, any conclusions obtained from the earlier reported results on the possible connection between observed DNA adduct levels and diet or disease status would be too precarious at the time being. A more extensive discussion on DNA adduct profiling and its link to diet and disease status will therefore be conducted during extensive follow-up studies. Nonetheless, the described DNA adductomics method already proved its usefulness as both targeted and putative untargeted diet-related DNA adducts were detected easily *in vivo*. Moreover, this is the first paper to report DNA adductome mapping of (tumoral) colonic tissue.

To provide an overview, the envisioned workflow for DNA adduct profiling in future DNA adductomic studies by means of the reported UHPLC-HRMS(/MS) method is documented in the graphical abstract.

4.4 The merit of a DNA adduct database

The human diet is quite complex and person-dependent, which implies that the impact of the individual human diet is very hard to pin to down. As diet and lifestyle significantly contribute to colon cancer risk, further investigation is warranted to link dietary toxicity to chronic disease through DNA adduct formation. Different diet-related DNA adduct types can be formed by e.g.

alkylation and oxidation or mycotoxin, HCA, PAH, and acrylamide exposure. In this context, a total of 123 different DNA adducts have currently been listed in an in-house (diet-related) DNA adduct database. Most of the listed DNA adducts have already been investigated *in vitro* or *in vivo* in relation to one or multiple 'suspicious' foodstuffs (see table 3 for a more detailed account and references). As the currently available information concerning the genotoxic effects of different food constituents is quite elaborate, yet still inconclusive, and since the use of DNA adductomics is still under development, it may be stated that although our DNA adduct list (anno 2015; table 3) is already quite extensive, it is not definitive. Nevertheless, due to the complex nature of the human diet and the long list of related research questions, the database can definitely help expose relevant DNA adduct types that require a more extensive investigation.

As different heterocyclic amine, polycyclic aromatic hydrocarbon, mycotoxin and acrylamide generated DNA adducts were also included in the database, the untargeted UHPLC-HRMS approach and database can also be put to use in related research that focuses on other diet-related chronic diseases. Furthermore, the self-constructed database includes a multitude of DNA adducts generated by unspecific alkylation and oxidation reactions which could be caused by several types of genotoxic, mutagenic and carcinogenic chemicals.

5. CONCLUSION

Hybrid HRMS systems yield highly specific information on compound mass, elemental composition and identity, whilst also enabling study of fragmentation patterns offering several advantages regarding structural elucidation. In consequence, such MS systems are particularly well suited for DNA adductomics [12, 72]. As it is our goal to implement the described method in the search for DNA adduct formation through dietary exposure to genotoxic chemicals in the meat-cancer relationship, the newly developed and successfully validated UHPLC-HRMS(/MS) method currently combines the simultaneous detection of 4 structurally and chemically different DNA adducts. The obtained LODs and LOQs for the targeted detection of O^6-CMG, O^6-MeG, M_1G and CroG with SIM-MS/MS allow *in vitro* and *in vivo* application. The state-of-the-art hybrid MS method also showed great promise for untargeted DNA adduct detection in future DNA adductomic studies as the Orbitrap revealed the presence of several putatively identified

DNA adducts in different DNA samples. This confirms the applicability of the untargeted full scan HRMS approach and its envisioned use for DNA adductome mapping. The use of the in-house DNA-adduct database in research focusing on diet and lifestyle related chronic diseases could expedite exposure of relevant biomarkers and provide new insights in disease etiology and prevention. The described adductomics methodology and database will thus serve as a basis for the analysis of both endogenous and exogenous DNA adducts in DNA from several tissue types and for a wide variety of research topics.

REFERENCES

[1] M.C. Poirier, Chemical-induced DNA damage and human cancer risk, Nat Rev Cancer, 4 (2004) 630-637.
[2] P.B. Farmer, DNA and protein adducts as markers of genotoxicity, Toxicology letters, 149 (2004) 3-9.
[3] R. De Bont, N. van Larebeke, Endogenous DNA damage in humans: a review of quantitative data, Mutagenesis, 19 (2004) 169-185.
[4] G.S. Qian, R.K. Ross, M.C. Yu, J.M. Yuan, Y.T. Gao, B.E. Henderson, G.N. Wogan, J.D. Groopman, A follow-up study of urinary markers of aflatoxin exposure and liver cancer risk in Shanghai, People's Republic of China, Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology, 3 (1994) 3-10.
[5] J. Angerer, U. Ewers, M. Wilhelm, Human biomonitoring: State of the art, International Journal of Hygiene and Environmental Health, 210 (2007) 201-228.
[6] G.G. Kuhnle, S.A. Bingham, Dietary meat, endogenous nitrosation and colorectal cancer, Biochemical Society transactions, 35 (2007) 1355-1357.
[7] V.L. Go, D.A. Wong, R. Butrum, Diet, nutrition and cancer prevention: where are we going from here?, The Journal of nutrition, 131 (2001) 3121s-3126s.
[8] D.E. Corpet, Red meat and colon cancer: should we become vegetarians, or can we make meat safer?, Meat science, 89 (2011) 310-316.
[9] L.J. Marnett, Oxy radicals, lipid peroxidation and DNA damage, Toxicology, 181-182 (2002) 219-222.
[10] M.W. Himmelstein, P.J. Boogaard, J. Cadet, P.B. Farmer, J.H. Kim, E.A. Martin, R. Persaud, D.E. Shuker, Creating context for the use of DNA adduct data in cancer risk assessment: II. Overview of methods of identification and quantitation of DNA damage, Critical reviews in toxicology, 39 (2009) 679-694.
[11] P.B. Farmer, R. Singh, Use of DNA adducts to identify human health risk from exposure to hazardous environmental pollutants: The increasing role of mass spectrometry in assessing biologically effective doses of genotoxic carcinogens, Mutation Research/Reviews in Mutation Research, 659 (2008) 68-76.
[12] S. Balbo, R.J. Turesky, P.W. Villalta, DNA adductomics, Chemical research in toxicology, 27 (2014) 356-366.

[13] H. Koc, J.A. Swenberg, Applications of mass spectrometry for quantitation of DNA adducts, Journal of Chromatography B, 778 (2002) 323-343.
[14] S. Balbo, S.S. Hecht, P. Upadhyaya, P.W. Villalta, Application of a High-Resolution Mass-Spectrometry-Based DNA Adductomics Approach for Identification of DNA Adducts in Complex Mixtures, Analytical Chemistry, 86 (2014) 1744-1752.
[15] J.M. Gavina, C. Yao, Y.L. Feng, Recent developments in DNA adduct analysis by mass spectrometry: a tool for exposure biomonitoring and identification of hazard for environmental pollutants, Talanta, 130 (2014) 475-494.
[16] B.H. Monien, Mass spectrometric DNA adduct quantification by multiple reaction monitoring and its future use for the molecular epidemiology of cancer, Advances in experimental medicine and biology, 806 (2014) 383-397.
[17] X. Liu, Z. Ser, J.W. Locasale, Development and Quantitative Evaluation of a High-Resolution Metabolomics Technology, Analytical chemistry, 86 (2014) 2175-2184.
[18] C. Xie, D. Zhong, K. Yu, X. Chen, Recent advances in metabolite identification and quantitative bioanalysis by LC-Q-TOF MS, Bioanalysis, 4 (2012) 937-959.
[19] J. Vanden Bussche, S.A. Moore, F. Pasmans, G.G. Kuhnle, L. Vanhaecke, An approach based on ultra-high pressure liquid chromatography-tandem mass spectrometry to quantify O6-methyl and O6-carboxymethylguanine DNA adducts in intestinal cell lines, Journal of chromatography. A, 1257 (2012) 25-33.
[20] E. Gottschalg, G.B. Scott, P.A. Burns, D.E. Shuker, Potassium diazoacetate-induced p53 mutations in vitro in relation to formation of O6-carboxymethyl- and O6-methyl-2'-deoxyguanosine DNA adducts: relevance for gastrointestinal cancer, Carcinogenesis, 28 (2007) 356-362.
[21] US Food and Drug Administration, Draft Guidance for Industry for Bioanalytical Method Validation, www.fda.gov, (2013).
[22] US Food and Drug Administration, Guidelines for the Validation of Chemical Methods for the FDA Foods Program, www.fda.gov, (2012).
[23] The Commision of the European Communities, Commission Decision of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results, eur-lex.europa.eu, (2002).
[24] A. Pfohl-Leszkowicz, Y. Grosse, V. Carriere, P.H. Cugnenc, A. Berger, F. Carnot, P. Beaune, I. de Waziers, High levels of DNA adducts in human colon are associated with colorectal cancer, Cancer Res, 55 (1995) 5611-5616.
[25] J. Vanden Bussche, L.Y. Hemeryck, T. Van Hecke, G.G. Kuhnle, F. Pasmans, S.A. Moore, T. Van de Wiele, S. De Smet, L. Vanhaecke, O(6)-carboxymethylguanine DNA adduct formation and lipid peroxidation upon in vitro gastrointestinal digestion of haem-rich meat, Molecular nutrition & food research, 58 (2014) 1883-1896.
[26] A.S. Jones, P. Lewis, S.F. Withers, The synthesis of carboxymethyl derivatives of purines and pyrimidines and their condensation with naturally occurring macromolecules, Tetrahedron, 29 (1973) 2293-2296.
[27] B. Lesyng, W. Saenger, Theoretical investigations on the conformation of 1,5,N(4),N(4)-tetramethylcytosine, Biochimica et biophysica acta, 544 (1978) 215-224.
[28] A. Segal, J.J. Solomon, U. Maté, Isolation of 3-(2-carboxyethyl)thymine following in vitro reaction of β-propiolactone with calf thymus DNA, Chemico-Biological Interactions, 29 (1980) 335-346.
[29] P.J. Aduma, S.V. Gupta, A.L. Stuart, G. Tourigny, Regulatory effects of deoxyribonucleosides on the activity of 5-methoxymethyl-2'-deoxycytidine: modulation of antiherpes activity by deoxyguanosine and tetrahydrodeoxyuridine, Antiviral research, 15 (1991) 301-313.

[30] N.B. Perry, J.W. Blunt, M.H.G. Munro, 1,3,7-Trimethylguanine from the Sponge Latrunculia brevis, Journal of Natural Products, 50 (1987) 307-308.
[31] L. Airoldi, C. Magagnotti, M. Bonfanti, L. Chiappetta, M. Lolli, C. Medana, G. De Gregorio, R. Fanelli, Detection of O6-butyl- and O6-(4-hydroxybutyl)guanine in urothelial and hepatic DNA of rats given the bladder carcinogen N-nitrosobutyl(4-hydroxybutyl)amine, Carcinogenesis, 15 (1994) 2297-2301.
[32] A. Kopplin, G. Eberle-Adamkiewicz, K.-H. Glüsenkamp, P. Nehls, U. Kirstein, Urinary excretion of 3-methyladenine and 3-ethyladenine after controlled exposure to tobacco smoke, Carcinogenesis, 16 (1995) 2637-2641.
[33] C. Zhao, M. Tyndyk, I. Eide, K. Hemminki, Endogenous and background DNA adducts by methylating and 2-hydroxyethylating agents, Mutation research, 424 (1999) 117-125.
[34] D. Palli, C. Saieva, C. Coppi, G. Del Giudice, C. Magagnotti, G. Nesi, F. Orsi, L. Airoldi, O6-alkylguanines, dietary N-nitroso compounds, and their precursors in gastric cancer, Nutrition and cancer, 39 (2001) 42-49.
[35] L.J. Marnett, Oxyradicals and DNA damage, Carcinogenesis, 21 (2000) 361-370.
[36] F. Drabløs, E. Feyzi, P.A. Aas, C.B. Vaagbø, B. Kavli, M.S. Bratlie, J. Peña-Diaz, M. Otterlei, G. Slupphaug, H.E. Krokan, Alkylation damage in DNA and RNA—repair mechanisms and medical significance, DNA Repair, 3 (2004) 1389-1407.
[37] P.J. Brooks, J.A. Theruvathu, DNA adducts from acetaldehyde: implications for alcohol-related carcinogenesis, Alcohol, 35 (2005) 187-193.
[38] H.A. Billson, K.L. Harrison, N.P. Lees, C.N. Hall, G.P. Margison, A.C. Povey, Dietary variables associated with DNA N7-methylguanine levels and O6-alkylguanine DNA-alkyltransferase activity in human colorectal mucosa, Carcinogenesis, 30 (2009) 615-620.
[39] W.Y. Hsu, W.T. Chen, W.D. Lin, F.J. Tsai, Y. Tsai, C.T. Lin, W.Y. Lo, L.B. Jeng, C.C. Lai, Analysis of urinary nucleosides as potential tumor markers in human colorectal cancer by high performance liquid chromatography/electrospray ionization tandem mass spectrometry, Clinica chimica acta; international journal of clinical chemistry, 402 (2009) 31-37.
[40] G. Cheng, M. Wang, P.W. Villalta, S.S. Hecht, Detection of 7-(2'-Carboxyethyl)guanine but Not 7-Carboxymethylguanine in Human Liver DNA, Chemical research in toxicology, 23 (2010) 1089-1096.
[41] H.-i. Kang, C. Konishi, T. Kuroki, N.-h. Huh, Detection of O6-methylguanine, O4-methylthymine and O4-ethylthymine in human liver and peripheral blood leukocyte DNA, Carcinogenesis, 16 (1995) 1277-1280.
[42] B.N. Ames, Endogenous DNA damage as related to cancer and aging, Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 214 (1989) 41-46.
[43] L.J. Marnett, Lipid peroxidation-DNA damage by malondialdehyde, Mutation research, 424 (1999) 83-95.
[44] F.L. Chung, J. Pan, S. Choudhury, R. Roy, W. Hu, M.S. Tang, Formation of trans-4-hydroxy-2-nonenal- and other enal-derived cyclic DNA adducts from omega-3 and omega-6 polyunsaturated fatty acids and their roles in DNA repair and human p53 gene mutation, Mutation research, 531 (2003) 25-36.
[45] M.S. Cooke, M.D. Evans, M. Dizdaroglu, J. Lunec, Oxidative DNA damage: mechanisms, mutation, and disease, The FASEB Journal, 17 (2003) 1195-1214.
[46] M. Valko, M. Izakovic, M. Mazur, C. Rhodes, J. Telser, Role of oxygen radicals in DNA damage and cancer incidence, Mol Cell Biochem, 266 (2004) 37-56.
[47] S.H. Lee, J.A. Arora, T. Oe, I.A. Blair, 4-Hydroperoxy-2-nonenal-induced formation of 1,N2-etheno-2'-deoxyguanosine adducts, Chemical research in toxicology, 18 (2005) 780-786.

[48] U. Nair, H. Bartsch, J. Nair, Lipid peroxidation-induced DNA damage in cancer-prone inflammatory diseases: A review of published adduct types and levels in humans, Free Radical Biology and Medicine, 43 (2007) 1109-1120.
[49] D. Gasparutto, E. Muller, S. Boiteux, J. Cadet, Excision of the oxidatively formed 5-hydroxyhydantoin and 5-hydroxy-5-methylhydantoin pyrimidine lesions by Escherichia coli and Saccharomyces cerevisiae DNA N-glycosylases, Biochimica et biophysica acta, 1790 (2009) 16-24.
[50] A. Valavanidis, T. Vlachogianni, C. Fiotakis, 8-hydroxy-2' -deoxyguanosine (8-OHdG): A critical biomarker of oxidative stress and carcinogenesis, Journal of environmental science and health. Part C, Environmental carcinogenesis & ecotoxicology reviews, 27 (2009) 120-139.
[51] P.-H. Chou, S. Kageyama, S. Matsuda, K. Kanemoto, Y. Sasada, M. Oka, K. Shinmura, H. Mori, K. Kawai, H. Kasai, H. Sugimura, T. Matsuda, Detection of Lipid Peroxidation-Induced DNA Adducts Caused by 4-Oxo-2(E)-nonenal and 4-Oxo-2(E)-hexenal in Human Autopsy Tissues, Chemical research in toxicology, 23 (2010) 1442-1448.
[52] R.J. Turesky, Formation and biochemistry of carcinogenic heterocyclic aromatic amines in cooked meats, Toxicology letters, 168 (2007) 219-227.
[53] R.J. Turesky, P. Vouros, Formation and analysis of heterocyclic aromatic amine–DNA adducts in vitro and in vivo, Journal of Chromatography B, 802 (2004) 155-166.
[54] H.A.J. Schut, E.G. Snyderwine, DNA adducts of heterocyclic amine food mutagens: implications for mutagenesis and carcinogenesis, Carcinogenesis, 20 (1999) 353-368.
[55] I.L. Steffensen, J.E. Paulsen, J. Alexander, The food mutagen 2-amino-9H-pyrido[2,3-b]indole (AalphaC) but not its methylated form (MeAalphaC) increases intestinal tumorigenesis in neonatally exposed multiple intestinal neoplasia mice, Carcinogenesis, 23 (2002) 1373-1378.
[56] D.H. Phillips, Polycyclic aromatic hydrocarbons in the diet, Mutation research, 443 (1999) 139-147.
[57] B. Schoket, DNA damage in humans exposed to environmental and dietary polycyclic aromatic hydrocarbons, Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 424 (1999) 143-153.
[58] J.S. Wang, J.D. Groopman, DNA damage by mycotoxins, Mutation research, 424 (1999) 167-181.
[59] I.R. McConnell, R.C. Garner, DNA adducts of aflatoxins, sterigmatocystin and other mycotoxins, IARC Sci Publ, (1994) 49-55.
[60] G. Gamboa da Costa, M.I. Churchwell, L.P. Hamilton, L.S. Von Tungeln, F.A. Beland, M.M. Marques, D.R. Doerge, DNA Adduct Formation from Acrylamide via Conversion To Glycidamide in Adult and Neonatal Mice, Chemical research in toxicology, 16 (2003) 1328-1337.
[61] A. Besaratinia, G.P. Pfeifer, DNA adduction and mutagenic properties of acrylamide, Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 580 (2005) 31-40.
[62] I. Manière, T. Godard, D.R. Doerge, M.I. Churchwell, M. Guffroy, M. Laurentie, J.-M. Poul, DNA damage and DNA adduct formation in rat tissues following oral administration of acrylamide, Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 580 (2005) 119-129.
[63] M.H. Lewin, N. Bailey, T. Bandaletova, R. Bowman, A.J. Cross, J. Pollock, D.E.G. Shuker, S.A. Bingham, Red Meat Enhances the Colonic Formation of the DNA Adduct O6-Carboxymethyl Guanine: Implications for Colorectal Cancer Risk, Cancer Research, 66 (2006) 1859-1865.
[64] C.N. Hall, A.F. Badawi, P.J. O'Connor, R. Saffhill, The detection of alkylation damage in the DNA of human gastrointestinal tissues, British journal of cancer, 64 (1991) 59-63.
[65] B.C. Cupid, Z. Zeng, R. Singh, D.E. Shuker, Detection of O6-carboxymethyl-2'-deoxyguanosine in DNA following reaction of nitric oxide with glycine and in human blood

DNA using a quantitative immunoslot blot assay, Chemical research in toxicology, 17 (2004) 294-300.
[66] Y.C. Jeong, J.A. Swenberg, Formation of M1G-dR from endogenous and exogenous ROS-inducing chemicals, Free radical biology & medicine, 39 (2005) 1021-1029.
[67] S.S. Mirvish, A.E. Ross, B. Gold, N. Drake, In vitro and in vivo formation of 7-(2'-carboxyethyl)guanine from the liver carcinogen 1-nitroso-5,6-dihydrouracil and its reactions with water and methanol, Journal of the National Cancer Institute, 74 (1985) 1105-1110.
[68] B. Tudek, E. Speina, Oxidatively damaged DNA and its repair in colon carcinogenesis, Mutation research, 736 (2012) 82-92.
[69] D.E.G. Shuker, G.P. Margison, Nitrosated Glycine Derivatives as a Potential Source of O6-Methylguanine in DNA, Cancer Research, 57 (1997) 366-369.
[70] H. Bartsch, J. Nair, Chronic inflammation and oxidative stress in the genesis and perpetuation of cancer: role of lipid peroxidation, DNA damage, and repair, Langenbeck's archives of surgery / Deutsche Gesellschaft fur Chirurgie, 391 (2006) 499-510.
[71] W. Lijinsky, N-Nitroso compounds in the diet, Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 443 (1999) 129-138.
[72] N. Tretyakova, P.W. Villalta, S. Kotapati, Mass Spectrometry of Structurally Modified DNA, Chemical Reviews, 113 (2013) 2395-2436.

CHAPTER IV

Shifts in the *in vitro* DNA adductome due to the digestion of red *vs.* white meat, and addition of calciumcarbonate

Adapted from:

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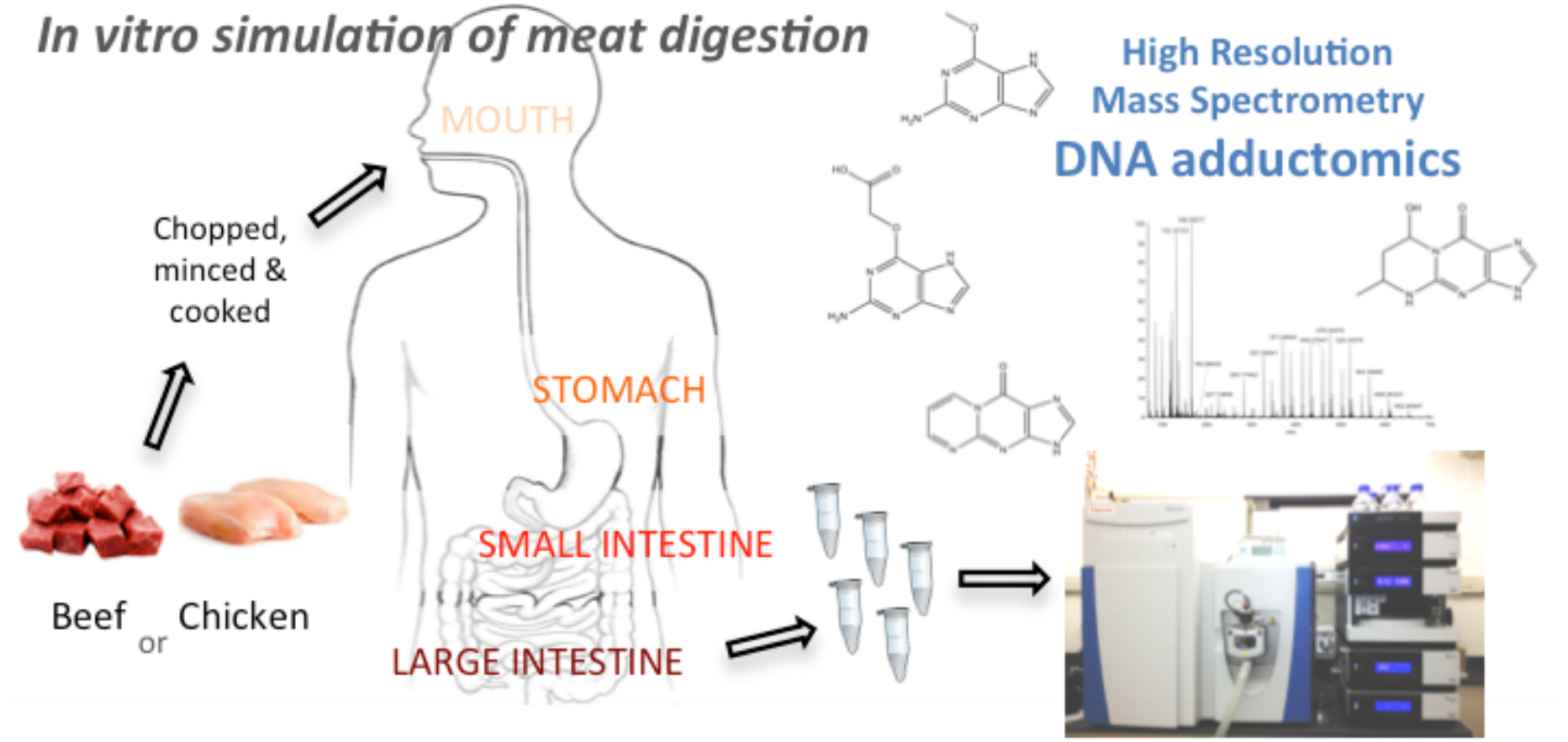
ABSTRACT

CRC is the third most common cancer type in the world. Epidemiological research has demonstrated that both red and processed meat consumption significantly contribute to CRC risk. In this study, red meat toxicity was investigated by means of simulated gastrointestinal conditions, MDA analysis and UHPLC-(HR)MS(/MS) based DNA adductomics. Since dairy products with high calcium content are associated with a decreased CRC-risk, the possible CRC-protective effects of calcium were assessed as well. The obtained results confirmed the earlier reported finding that heme-rich meat stimulates lipid peroxidation and O^6-CMG DNA adduct formation during digestion. Calcium carbonate ($CaCO_3$) supplementation resulted in both toxic and anti-toxic effects; i.e. stimulation of O^6-CMG production, but reduction of MDA formation. DNA adductome mapping of meat digests revealed a significant interindividual variability. The observed DNA adduct profile also differed according to the digested meat type, uncovering different putative DNA adducts that seem to be associated with digestion of beef or chicken with or without supplemented $CaCO_3$. Formamidopyrimidine-adenine was found to be discriminative for meat digests without added $CaCO_3$, carboxyethylcytosine was significantly higher in beef digests and methoxymethylcytosine (or its hydroxyethylcytosine isomer) was found to be lower in meat digests supplemented with $CaCO_3$. These results demonstrate that DNA adduct formation may be involved in the pathway that links red meat digestion to CRC promotion. In addition, the possible CRC-protective attributes of calcium through anti-oxidant actions could be documented.

Keywords:

Calcium, Cancer Risk, DNA Adductome Mapping, Malondialdehyde,
O^6-Carboxymethylguanine, Red Meat Digestion

GRAPHICAL ABSTRACT



1. INTRODUCTION

With increasing age, both men and women are often afflicted with CRC, the third most common cancer type in men and the second most common in women [1]. The vast majority of CRC cases (about 90 %) and deaths (about 70 %) can be attributed to environmental factors, with diet being the most significant contributor [2]. Since CRC incidence is higher in economically developed and developing countries [3], epidemiologists have looked for evidence explaining a possible causal link between the consumption of typical Western foodstuffs and CRC risk. Their results suggest that the 'excessive' consumption of red and/or processed meat may involve significant adverse health effects [4, 5]. Although there is no mistaking that meat does provide us with essential nutrients, the current hypothesis on red meat carcinogenicity states that heme, which is more present in red meat compared to white meat, may be responsible for the increase in red meat associated CRC incidence due to certain indirect geno- and cytotoxic effects. The "heme hypothesis" states that this molecule stimulates the formation of LPOs and NOCs in the gut, which is why both LPOs and NOCs have been linked to the hypothesis on red meat associated CRC initiation and/or promotion [4, 5].

LPOs are formed when reactive oxygen species and iron (Fe) interact with lipids like polyunsaturated fatty acids, resulting in the formation of different (highly) reactive electrophilic epoxides and aldehydes [6]. One of the major LPOs is MDA and additional examples of lipid peroxidation end products include acetaldehyde, hydroxynonenal and CRO [7, 8]. Several of these LPOs have known cyto- and genotoxic effects that may lead to mutagenesis and carcinogenesis via the formation of promutagenic DNA adducts [8]. Several *in vivo* studies have already demonstrated a significant increase in both LPOs and LPO derived DNA adducts in 'normal tissue' due to oxidative stress and/or dietary imbalance, as well as in different human malignancies [6, 9].

NOCs are potent carcinogens that can induce DNA adduct formation (e.g. O^6-MeG and O^6-CMG) due to their alkylating properties [10, 11]. Diet represents the most important route of NOC exposure [12] although NOCs can also be formed endogenously in the stomach (interaction of nitric oxide or nitrite and secondary or tertiary amines) [12, 13] and large bowel (nitrosation of amines after microbial fermentation of proteins in the gut) [14, 15]. More importantly, an increase in NOC uptake has been linked to an increased consumption of red

meat and an increase in the levels of a specific alkylation induced DNA adduct (O^6-CMG) in colonic tissue at least once before [11].

The list of different food related genotoxic compounds that may induce DNA adduct formation appears to be quite extensive, as is the collection of possible resulting DNA adducts [16]. Since attack of DNA nucleobases by environmental toxins can indeed lead to DNA mutations, chromosomal alterations and chemically induced carcinogenesis [17, 18], the need to investigate the prevalence of different types of diet-related DNA adducts in meat digests has presented itself. Some of our previous work confirmed a rise in (MDA and) O^6-CMG DNA adduct levels upon digestion of heme-rich red meat [19, 20]; a process that appears to be stimulated in the event of digestion of overcooked meat or meat with a high fat content [21, 22]. Two complimentary studies by Winter et al. [23, 24] investigated the formation of hydroxyguanine (an extensively studied oxidative DNA adduct) and O^6-MeG in mice, revealing that red meat and/or heme can induce a rise in the formation of those particular DNA adducts. In addition, as was already mentioned before, Lewin et al. [11] reported the presence of higher O^6-CMG levels in human colonic cells after red meat consumption. To be able to investigate the current hypothesis on red meat induced LPO and NOC related DNA adduct formation, we aimed to further examine the possible genotoxic effects of red meat consumption and digestion by means of the *in vitro* gastrointestinal digestion of meat and a recently developed DNA adductomics platform [25]. This will allow us to analyze the presence of both targeted and untargeted diet-related DNA adducts in meat digests and enable a more in-depth investigation of the possible underlying pathway(s) of red meat associated genotoxicity, and the possible protective attributes of calcium.

2. EXPERIMENTAL

2.1 Meat preparations

Four different types of meat preparations were established; beef with and without addition of $CaCO_3$, and chicken with and without addition of $CaCO_3$. Beef diaphragm (high heme iron content; as a model for red meat), chicken breast (low heme iron content; as a model for white meat) and subcutaneous pork fat were obtained from a local slaughterhouse and butcher. The beef and chicken samples were chopped into cubes (1 to 2 cm^3) separately, and pork fat was

added to each of them in order to obtain a total fat content of 20 %. The meat samples were minced (omega T-12 (Omega Foodtech, Bologna, Italy) equipped with a 10 mm plate) and grounded (by means of a 3.5 mm plate) thoroughly. Subsequently, all meat preparations were heated for 30 min after reaching a core temperature of 90°C in a hot water bath (GFL, Grossburgwedel, Germany). As a final step, meat preparations were homogenized with a food processor and stored at -20°C. To obtain meat preparations supplemented with calcium, a solution of $CaCO_3$ was freshly added (0.025 g $CaCO_3$ per g meat) just before the start of the *in vitro* gastrointestinal digestion simulations.

2.2 *In vitro* gastrointestinal digestion of meat

2.2.1 Experimental setup

In this study, two different types of experiments were set up, a first experiment to screen for DNA adduct formation upon *in vitro* beef digestion using the fecal microbiota of 5 different individuals and a more extensive (second) experiment to compare DNA adduct formation during and after digestion of different meat type preparations. The first experiment entailed 5 separate *in vitro* gastrointestinal digestions of beef during which the fecal inocula (see 2.2.2 Collection, storage and precultivation of colonic microbiota) of 5 different volunteers were used to simulate colonic meat digestion. In the follow-up experiment, 3 different fecal inocula obtained from 2 out of 5 initial volunteers (see 2.2.2 Collection, storage and precultivation of colonic microbiota) were employed to ferment the four meat preparations (beef without added $CaCO_3$, beef with added $CaCO_3$, chicken without added $CaCO_3$ and chicken with added $CaCO_3$) in the *in vitro* gastrointestinal setup in triplicate, resulting in 3 biological (by means of 3 different fecal inocula obtained from 2 out of 5 initial volunteers) and 3 technical replicate digestions for each meat preparation (all performed in separate vessels, resulting in a total of 36 separate meat digestions).

2.2.2 Collection, storage and precultivation of colonic microbiota

For initial screening, fresh fecal samples were obtained from 5 male volunteers on a Western-type diet (uncontrolled and unmonitored) with no medical history of gastrointestinal disease. None of the volunteers had received antibiotic treatment during at least 6 months prior to

donation. For a follow-up experiment, 3 new fecal samples were obtained from 2 out of 5 volunteers at different points in time (interval > 7 days). The 5 human donors of fecal material were recruited among the laboratory personnel through informal announcement. All volunteers have given their written informed consent. The obtained data and volunteer information were analyzed anonymously and de-identified. The research was approved by the Federal Public Service of Health, Food Chain Safety and Environment, Belgium, but there was no need to submit an application to the ethical committee due to the non-invasive nature of the voluntary donation of fecal samples.

All fecal samples were processed according to a protocol adapted from Molly et al. [20, 26] Fresh fecal material was diluted in preheated PBS solution (1:4; w/v), to which sodium thioglycolate (1 g/L) was added as a reducing agent. The hence obtained fecal slurry was then filtered through a 1 mm metal sieve and stored at -80°C on a glycerol stock (20 %). After storage and prior to use for the gastrointestinal digestion of meat, anaerobic precultivation of fecal inocula in BHI broth (obtained from Oxoid Ltd, Hampshire, GB) with added cysteine (37 g/L BHI + 0.5 g/L cysteine) at a 1:9 ratio (v/v)) was performed at 37°C for 24 h.

2.2.3 Stomach, small bowel and large bowel digestion simulation

The *in vitro* gastrointestinal digestion model enabled a consecutive simulation of stomach, small and large bowel digestion. For the treatments with calcium, a solution of $CaCO_3$ was freshly added (0.025 g $CaCO_3$ per g meat) just prior to the *in vitro* digestion. During the first digestion step, 6 mL of simulated saliva and 12 mL of simulated gastric juice were added to 4.5 g of meat (beef without added $CaCO_3$, beef with added $CaCO_3$, chicken without added $CaCO_3$ or chicken with added $CaCO_3$). All were incubated under constant stirring (150 rpm) during 2 h at 37°C, after which 2 mL of bicarbonate buffer, 12 mL of simulated duodenal juice and 6 mL of mimicked bile juice were added to perform small bowel digestion (2 h at 37°C, stirred at 150 rpm). Colonic fermentation (48 h, 37°C, 150 rpm) in the large bowel was simulated by addition of SHIME medium (22 mL) and precultivated fecal inocula (22 mL) containing the colonic microbiota [26]. To ensure anaerobic conditions during colonic fermentation, 30 min of N_2-flushing was performed prior to incubation [20, 26]. Samples of meat digests were taken after simulation of duodenal digestion ("T0" samples, whereby sampling took place immediately after addition of SHIME medium and the fecal inoculum) and at the end of the simulated colonic

meat digestion ("T48" samples, whereby sampling took place after 48 h incubation with SHIME medium and fecal inocula). Samples were stored at -80°C awaiting analysis. For technical and practical matters concerning this gastro-intestinal digestion model and more details on preparation of all mimicked gastrointestinal juices, brain heart infusion broth and SHIME medium, we refer to previously reported research [19].

2.3 Sample preparation for DNA adduct analysis; DNA hydrolysis and DNA adduct extraction

DNA adducts in meat digests were extracted and purified according to the protocol of Vanden Bussche et al. [27] During the described protocol, O^6-d_3-methylguanine (Toronto Research Chemicals Inc., Toronto, Canada) was added as an internal standard, after which DNA samples were subjected to DNA hydrolysis (30 min, 80°C) in 0.1 M formic acid in water to cleave both adducted and non-adducted DNA nucleobases from all DNA sequences present. Following this, sample purification and cleanup was performed with SPE (Oasis® HLB cartridges (1 cc, 30 mg) Waters (Milford, USA)). After SPE, the collected samples were evaporated to dryness under vacuum (90 min, 20°C). Finally, all samples were suspended in 100 µL of 0.05 % of acetic acid in water and stored at -20°C until analysis.

2.4 Untargeted DNA adductomics

2.4.1 UHPLC-HRMS DNA adduct analysis

Analysis of DNA adducts in meat digests was effectuated by ultrahigh performance liquid chromatography coupled to UHPLC-HRMS. In brief, chromatographic separation was achieved with an Acquity BEH C18 Waters column (1.7 µm, 2.1 x 100 mm; Waters Corporation, Milford, USA) and the use of 0.05 % of acetic acid in water and 100 % methanol as mobile phases. The mobile phases were pumped at 300 µL per min by an Accela 1250 pump coupled to an Accela autosampler (Thermo Scientific, San José, USA). HRMS analysis was performed by means of an Orbitrap MS (Exactive™, Thermo Fisher Scientific, San José, USA) coupled to a HESI-II source. All MS and HESI settings are documented in table 1.

Table 1. HESI and MS settings for DNA adduct analysis.

Setting	Full MS
m/z scan range	70-700 Da
Mass resolution	100,000 Full Width Half Maximum
Ionization mode	Polarity switching
Maximum injection time	500 ms
Automatic gain target	3 x e^6
Spray voltage	(+/-) 4 kV
Sheath gas flow rate	35 arbitrary units
Auxiliary gas flow rate	5 arbitrary units
Sweep gas flow rate	2 arbitrary units
Capillary temperature	380°C
Heater temperature	370°C
Capillary voltage	(+/-) 40 V
Tube lens voltage	(+/-) 70 V
Skimmer voltage	(+/-) 15 V

Internal calibration of the MS system was performed daily by infusion of calibration mixtures prepared according to the protocol described in the operations manual (Thermo Fisher Scientific, San José, USA). General instrument control and initial data processing were performed with Xcalibur™ 2.1.

2.4.2 Data processing and statistics

2.4.2.1 ToxID™ profiling

By means of an in-house DNA adduct database25 and the use of ToxID™ software (Thermo Fisher Scientific, San José, USA), the full scan HRMS spectra of the meat digestion samples were screened for the possible presence of known diet-related DNA adducts. The inclusion criteria consisted of a minimum signal intensity of 10 000, a maximum mass deviation of 10 ppm, recurrence and stable retention time (RT) of the DNA adduct of interest in replicate samples and a C^{12}/C^{13} ratio approaching the natural 99:1 ratio.

2.4.2.2 Sieve™ data processing

2.4.2.2.1 Chromatographic peak selection with database lookup

To screen for the presence and significance of known DNA adducts in the different digested meat samples, two sample differential analysis was performed with the database lookup function of Sieve™ 2.1 (Thermo Fisher Scientific, San José, USA). This approach allows pairwise comparison of DNA adduct levels in different meat preparations and correct statistical interpretation and evaluation of the obtained results. Combining the spectral data obtained from all three biological replicate digestion samples from the second experiment assured repeatability of the obtained results in different technical and biological replicates of different individuals. The chosen parameter settings enabled retention of ions demonstrating an m/z value between 70 and 700 Da, also demonstrating an RT between 0.7 and 5.5 min (of chromatographic analysis). A mass deviation up to 10 ppm was allowed and the maximum peak width consisted of 0.5 min. Positive and negative ions were analyzed separately. The maximum number of frames and minimal peak intensity was set at 50 000 and 35 000 (frame number and arbitrary units respectively) for positive ion mode and 50 000 and 20 000 (frame number and arbitrary units, respectively) for negative ion mode. After automated processing, Sieve™ reported the m/z value and RT for each detected ion, also documenting on the signal abundance in each sample. In a subsequent step, the 'Database Lookup' and an in-house diet-related DNA adduct database were used for putative identification of the detected and selected ions.

2.4.2.2.2 Chromatographic peak selection in preparation of Simca™ analysis

In preparation of statistical analysis by means of Simca™, Sieve™ can be operated to select all detected chromatographic peaks. To this purpose, the same Sieve™ settings as described in 2.4.2.2.1 Chromatographic peak selection with database lookup, were applied, although the database lookup function was not enabled and the maximum number of frames and the minimal peak intensity (in arbitrary units) were both set at 35 000.

2.4.2.3 Simca™: orthogonal partial least squares discriminant analysis

Simca™ 13 (Umetrics AB, Umeå, Sweden) software can be implemented for Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) of multivariate omics data of which the

number of variables (X) exceeds the number of observations. During this type of analysis, a linear regression model that can fit a certain sample into a certain category (categorical Y) based on its X-variables, is constructed.

In this study, OPLS-DA was performed by the combined analysis of all raw data obtained from all 3 follow-up digestions for both selected test subjects separately, thus combining the data of all technical replicates (every experiment was performed in triplicate) for each biological replicate (3 per test subject; n = 9) for selected test subject 1 (P1) on the one hand and selected test subject 2 (P2) on the other.

After logarithmic data transformation (to ensure normal data distribution) and Pareto scaling (to limit large differences concerning the range of the different X-variables without leveling off), Simca™ allowed automated data modelling. The validity of the obtained OPLS-DA model was checked by CV-ANOVA ($p < 0.05$) and assessment of R^2 (must approach 1 (= perfect fit) and is used to assess the goodness of fit, representing the explained variation in X) and Q^2 (= cross-validated R^2, used to assess the predictive ability of the method and operated with a minimal threshold of 0.5). Discriminative/predictive ions were selected based on a Variable Importance in Projection-score (VIP score) > 0.8. The VIP score reflects the relative importance of X-variables in predicting the Y-variable. A VIP > 1 has a high influence, a VIP < 1, but > 0.8 has a moderate influence and a VIP < 0.8 has a low influence [28, 29].

2.5 Targeted analyses

2.5.1 O^6-carboxymethylguanine and O^6-methylguanine

Targeted analysis of the O^6-CMG and O^6-MeG DNA adducts was performed by means of UHPLC-MS/MS using a triple quadrupole MS (TSQ Vantage) coupled to a HESI-II source, an Accela pump and Accela autosampler (Thermo Fisher Scientific, San José, USA) [27]. Identification of O^6-CMG and O^6-MeG was achieved based on matching fragments and RT, derived from corresponding analytical standards. O^6-CMG was kindly provided by Dr. S. Moore (Liverpool John Moores University, Liverpool, UK), whilst O^6-MeG was purchased from Sigma-Aldrich (St Louis, MO, USA). Correct quantification was performed by means of a 12-point calibration curve in calf thymus DNA (Rockland, Gilbertsville, Pennsylvania, USA) [27].

Instrument control and data processing were performed with Xcalibur™ 2.1 (Thermo Fisher Scientific, San José, USA).

2.5.2 Malondialdehyde

MDA concentrations in meat digests were determined colorimetrically according to the protocol described by Van Hecke et al. [21], i.e. a slightly altered version of the TBARS protocol documented by Grotto et al. [30] MDA was allowed to react with 2-thiobarbituric acid in an acid environment, resulting in the formation of TBARS. After extraction of the formed TBARS by means of 1-butanol, the absorbance of the TBARS complexes was measured at 532 nm (G10S UV–Vis, Thermo Fisher Scientific, San José, USA). Quantification was enabled by means of an 8-point calibration curve (0 to 50 nmol of MDA per mL, constituted after acid and thermal lysis of 1,1,3,3-tetramethoxypropane (Sigma-Aldrich, St Louis, MO, USA)).

2.5.3 Statistics

Statistical analysis of the results obtained by means of all targeted analyses was performed with SAS enterprise guide 6 (SAS Institute Inc., Cary, North Carolina, USA), applying a type I error probability of 5 %.

3. RESULTS

3.1 DNA adduct profiling of *in vitro* beef digests using 5 different fecal inocula

Per test subject, individual *in vitro* gastrointestinal digestions of beef preparations were performed by adding its proper fecal inoculum to the colonic compartment. Samples for DNA adduct profiling purposes were collected directly after addition of pre-cultivated fecal inocula, but before colonic incubation (T0), and also after 48 hours of colonic digestion (T48), enabling assessment of interindividual differences over time.

Untargeted HRMS analysis was performed on the meat digests for all 5 test subjects. The acquired T0 and T48 sample spectra and chromatograms were screened for DNA adducts included in the diet-related database, thereby using ToxID™ data processing. This resulted in the putative identification of 46 untargeted DNA adducts, which were present at T0 and/or T48. To clearly visualize the increase or decrease of DNA adduct levels during *in vitro* colonic digestion of beef (difference in measured DNA adduct levels in T48 samples compared to T0 samples), measured putative DNA adduct signal intensities at T0 were subtracted from T48 DNA adduct signal intensities and visualized in a heat map (figure 1). In this heat map, positive values are colored red; meaning that the respective T48 signal intensity (for that compound and test subject) was higher than the T0 signal intensity, implying that DNA adduct levels increased during colonic digestion, whilst negative values are colored blue; meaning that T0 values were higher than T48 values, and that DNA adduct levels decreased during colonic digestion.

The detected levels of different putatively identified DNA adduct types increased during colonic digestion of beef, whilst others declined. None demonstrated a steady state. The detected DNA adduct types were somewhat similar for all tested fecal inocula, although significant interindividual differences could be noted in their intensity levels, both before (T0) and after (T48) colonic digestion of beef.

3.2 DNA adductomics in digests of different meat preparations

A second *in vitro* experiment was conducted using the fecal inocula of 2 out of the 5 previously employed volunteers. These particular volunteers (P1 & P2) were selected since their fecal inocula demonstrated a noticeable increase of O^6-CMG levels (RT 1.58 min) upon colonic digestion of beef. The production of O^6-CMG was of particular interest in this study since the *in vivo* occurrence of O^6-CMG has been linked to red meat digestion and may be involved in the development of CRC [11].

Meat preparations of beef and chicken, both with or without added $CaCO_3$, were subjected to 3 separate *in vitro* digestions as described in the Experimental section. Targeted O^6-CMG analysis and untargeted HRMS analysis was performed on the obtained samples at T0 and T48 to evaluate the effect of *in vitro* gastrointestinal digestion of different meat preparations on DNA adduct profile.

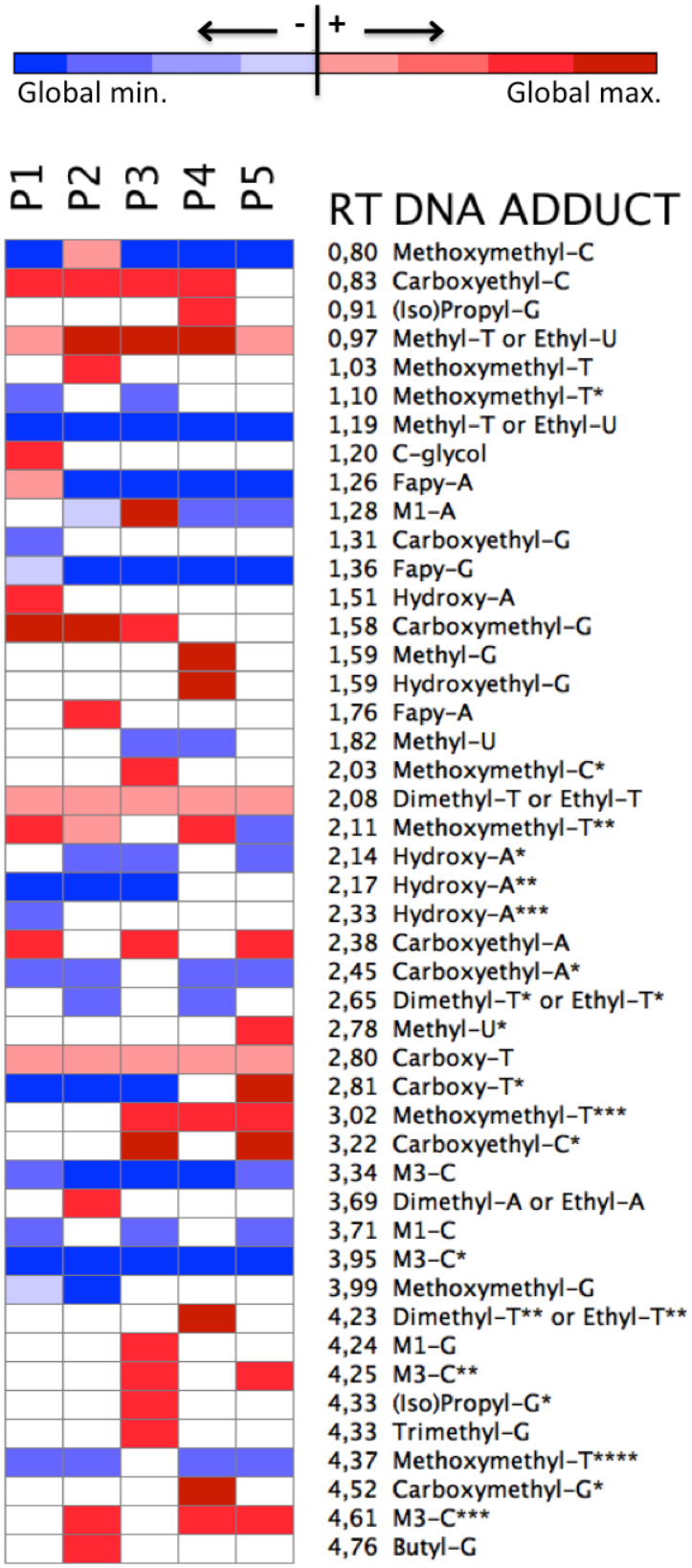


Figure 1. Heat map displaying rank transformed data (ToxID™) on increase (light to dark red) or decrease (light to dark blue) of DNA adduct levels (different isomers marked with *(*)(*)(*)) during 48 hours of colonic beef digestion (after stomach and small intestinal digestion) utilizing the fecal inocula of 5 different test subjects (P1, P2, P3, P4 and P5). To facilitate comparison, DNA adduct levels in T0 samples were subtracted from DNA adduct levels in T48 samples, rendering positive values to be red, and negative values to be blue. Darker shades document larger differences in DNA adduct signal intensity. White boxes represent DNA adducts that were not detected, Regarding the abbreviations in the DNA adduct names; G equals guanine, C equals cytosine, T equals thymine, A equals adenine and U stands for uracil; 'M' represents an adduct type originating from the reaction of a nucleobase with 1 or more MDA molecule(s). RT is expressed in min.

As this paper aims to further unravel the hypothesis on red meat related CRC risk, only tentatively identified DNA adduct types that were higher in red vs. white meat digests will be discussed in detail. Since calcium is believed to have CRC-protective attributes, the same applies for putative DNA adducts that appeared to be lower upon digestion of $CaCO_3$ supplemented meat.

3.2.1 DNA adduct profiling in digests of different meat preparations

The results of the DNA adduct analysis of digests of different meat preparations encompass the detection of several putatively identified DNA adducts. Figure 2 shows the DNA adduct types and levels in meat digests that were sampled after small intestinal digestion (= T0 samples). In figure 3, DNA adduct levels in digestive samples obtained after complete *in vitro* gastrointestinal digestion of all different meat preparations are compared (= T48 samples). The results demonstrate that certain DNA adduct types appear to be more prevalent in chicken digests compared to beef, whilst others were more common in beef digests. Inclusion of $CaCO_3$ in meat preparations induced a similar effect, resulting in higher or lower DNA adduct levels in those particular meat digests. Both figure 2 and figure 3 are heat maps that allow comparison of DNA adduct levels in different meat digests for a certain test subject (P1 or P2) by means of a color code. To enable this, the measured signal intensity for that particular DNA adduct in a specific meat digest type was subtracted from the measured signal intensity in a different meat digest type. An example, 'P1: B-C' allows comparison for DNA adduct levels in beef (B) and chicken (C) digests for test subject 1 (P1) as the detected signal intensity in chicken was subtracted from the observed signal intensity in beef. Since positive values in this heat map are marked red, and negative values are marked blue, a blue box would indicate that the obtained DNA adduct levels in chicken digests were higher compared to beef (B-C < 0). In contrast, a red box would signal higher DNA adduct levels in beef digests compared to chicken digests (B-C > 0).

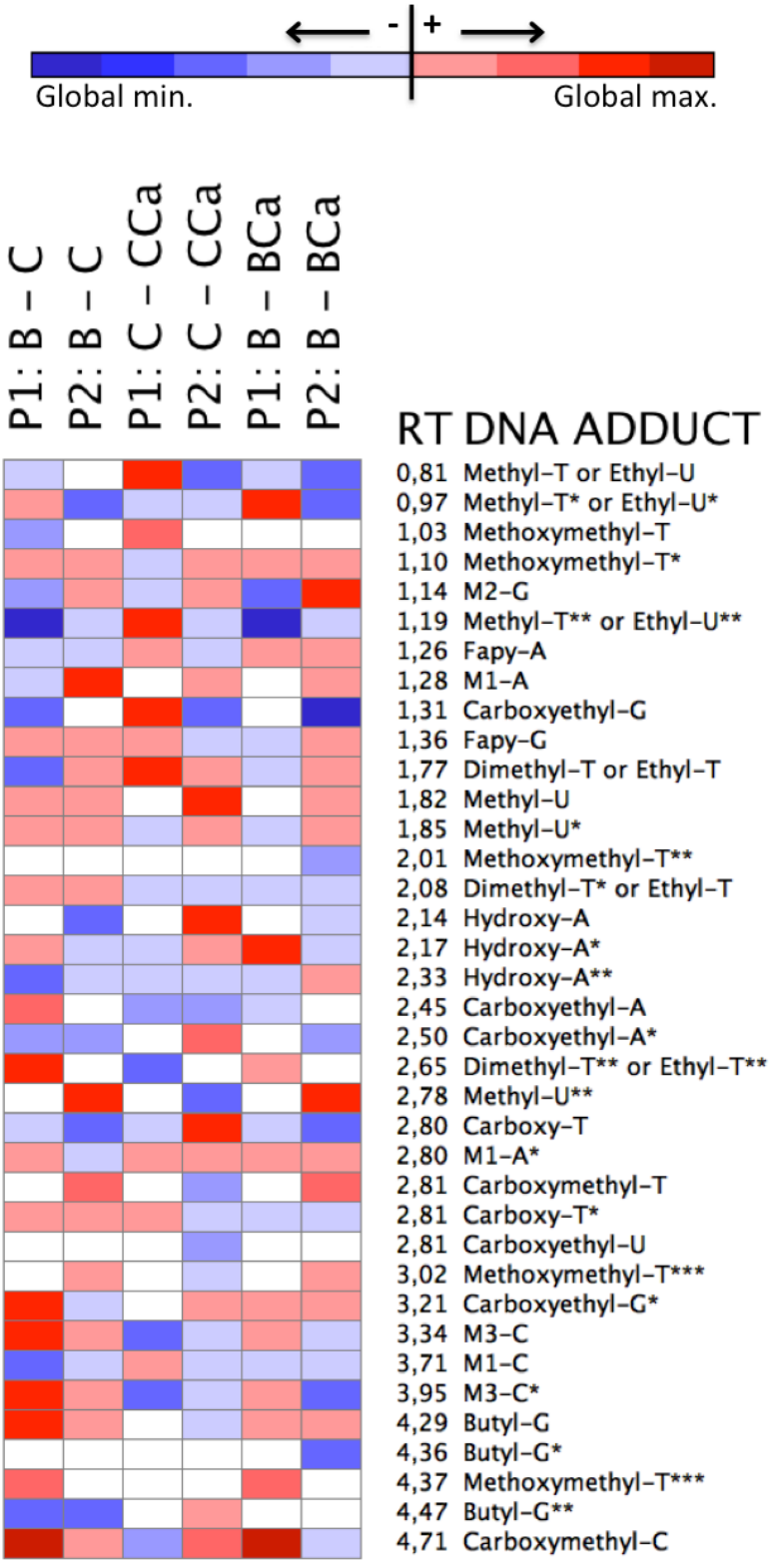


Figure 2. Heat map displaying rank transformed data (ToxID™) on average putatively detected DNA adduct levels (different DNA adduct isomers marked with *(*)(*)(*)) in T0 digestive samples by means of 3 separate *in vitro* setups utilizing 3 different fecal inocula of 2 different test subjects (P1 and P2) to compare DNA adduct levels and types in different meat preparation digests. White boxes imply the absence of that DNA adduct type in said sample. Red indicates higher putative DNA adduct levels in comparison (average DNA adduct level in the first noted meat digest minus the average DNA adduct level in the second noted meat digest). Blue represents lower putative DNA adduct levels in comparison. "B" represents beef digests, "C" represents chicken digests and "Ca" indicates the addition of $CaCO_3$ to the considered meat preparation that was digested. Concerning the DNA adduct name abbreviations; G equals guanine, C equals cytosine, T equals thymine, A equals adenine and U stands for uracil; 'M' represents an adduct type originating from the reaction of a nucleobase with 1 or more MDA molecule(s). RT is expressed in min.

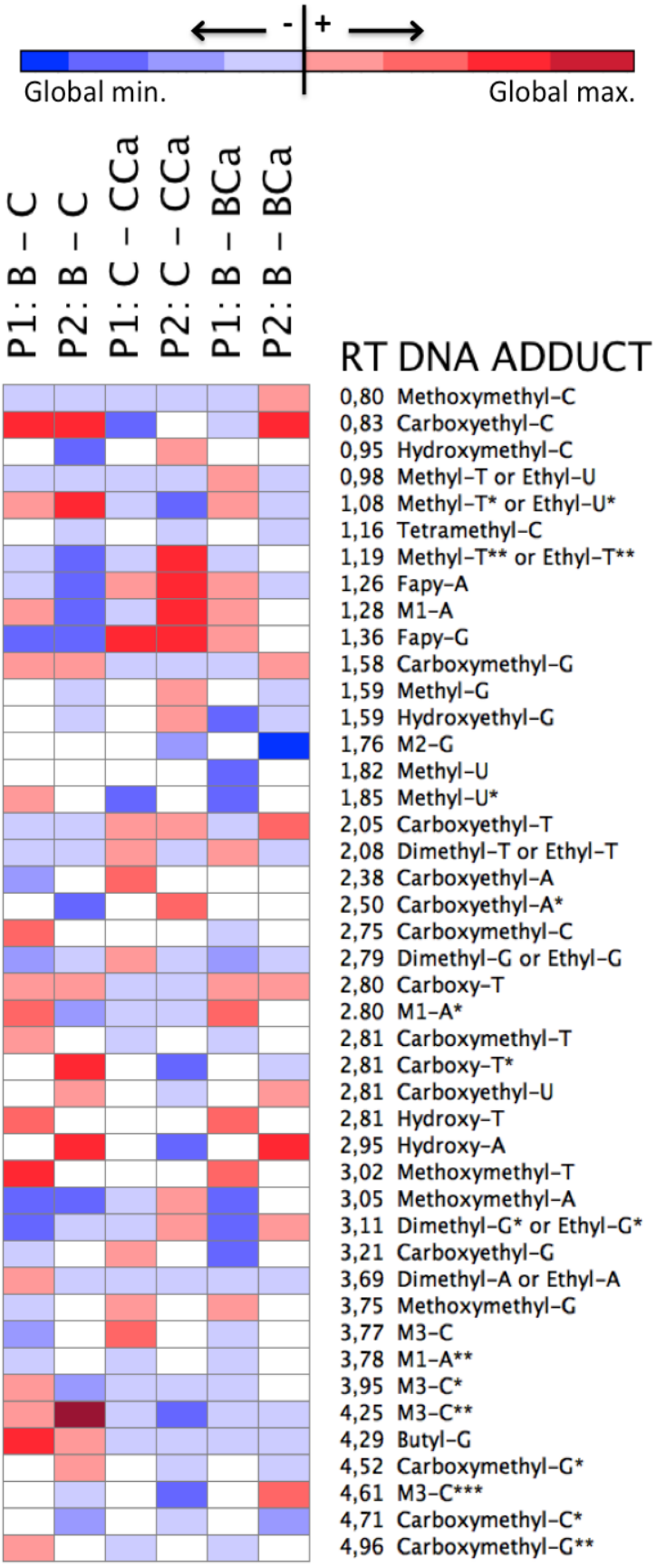


Figure 3. Heat map displaying average rank transformed ToxID™ data of putatively detected DNA adduct levels (different isomers marked with *(*)(*)(*)) in colonic digestive samples (T48) by means of 3 separate *in vitro* setups utilizing 3 different fecal inocula of 2 test subjects (P1 and P2) to compare DNA adduct levels and types in meat digests. White boxes represent the absence of measurable DNA adduct levels. Light to dark red represents higher putative DNA adduct levels in comparison (average DNA adduct level in the firstly mentioned digested meat preparation minus the average DNA adduct level in the secondly mentioned meat preparation digest). Light to dark blue represents lower putative DNA adduct levels in comparison. "B" stands for beef, "C" stands for chicken, and "Ca" stands for added $CaCO_3$. With regard to DNA adduct name abbreviations; G equals guanine, C equals cytosine, T equals thymine, A equals adenine and U stands for uracil; 'M' represents an adduct type originating from interaction with 1 or more MDA molecule(s). RT is expressed in min.

3.2.2 Two sample differential analysis by means of Sieve™ database lookup: DNA adducts in different meat preparations pre- and post-colonic digestion

DNA adduct types that were retrieved by means of two sample differential analysis of meat digest samples obtained after colonic digestion (at T48) are presented in table 2. Hereby, 7 different DNA adducts appeared to be significantly higher or lower in beef digests compared to chicken digests. Addition of $CaCO_3$ to beef or chicken did also result in a shifted DNA adduct profile. Just prior to colonic digestion (at T0), over 20 different DNA adduct types with significantly higher or lower levels in beef or chicken digests could be retrieved, whilst addition of $CaCO_3$ to chicken or beef also induced a significant increase or decrease in certain DNA adduct levels (compared to those in chicken or beef without added $CaCO_3$) (see table 3 and table 4). In the context of red meat genotoxicity, the putatively identified DNA adducts of interest are the ones with a significantly higher prevalence in beef digests. Regarding the possible CRC-protective attributes of calcium, investigation of the putatively identified DNA adducts that are lower in meat preparation digests with added calcium in comparison to digests of meat preparations without added calcium are prioritized. DNA adducts of interest that were retrieved at T0 are presented in table 3, whereas additional DNA adducts are documented in table 4.

3.2.3 Simca™ modelling; discrimination between digested meat preparations

OPLS-DA modelling of T48 sample composition did not reveal any discriminating DNA adducts for beef vs. chicken or added $CaCO_3$ vs. no added $CaCO_3$. However, 2 putative DNA adducts could be retained as discriminating factors for digestion of meat with added $CaCO_3$ in T0 samples. The putatively identified M_3C (diformyldihydromethano-oxazocinyloxopyrimidine, RT of 4.58 min and m/z of 300.0991 in negative ionization mode), a DNA adduct type resulting from the interaction of MDA and cytosine, was found to be discriminative for digestion of meat with added $CaCO_3$ (vs. meat without added $CaCO_3$) for P1. In contrast, fapy-A (4,6-diamino-5-formamidopyrimidine, RT of 1.14 min and m/z of 154.0723 in positive ionization mode), an oxidative adenine lesion, appeared to be predictive for the lack of $CaCO_3$ addition to meat preparations prior to *in vitro* digestion in P2 samples. The tentatively identified M_3C

demonstrated a VIP score of 0.732 in chicken digests and 0.815 in beef digests. Fapy-A demonstrated a VIP score of 1.78 in chicken digests and 2.70 in beef digests.

Table 2. Significantly higher levels ($p < 0.05$ or (*) $p < 0.10$) of putatively identified DNA adducts in colonic meat digests (T48) after two sample differential analysis by means of Sieve™ database lookup.

Discriminating for	DNA adduct	RT (min)
Beef *vs.* Chicken	Carboxyethyl-C	0.82
	Dimethyl-T or Ethyl-T	0.70
	M_3C (Diformyldihydromethano-oxazocinyloxopyrimidine)	4.12
Chicken *vs.* Chicken + $CaCO_3$	Methoxymethyl-C	0.78
	Methoxymethyl-C	0.80
	Methoxymethyl-C	1.03
	Methoxymethyl-C	1.30
	Methoxymethyl-C	1.59
	Methoxymethyl-C	1.84
	Methoxymethyl-C	2.18
Beef + $CaCO_3$ *vs.* Beef	3,N_4-etheno-C (*)	0.79
	Carboxymethyl-T (*)	0.74
	M_3C (Diformyldihydromethano-oxazocinyloxopyrimidine) (*)	1.07
	Methyl-G or Hydroxymethyl-A	1.45
	Methyl-T or Ethyl-U (*)	2.46
	Methyl-T or Ethyl-U	3.19
	Tetramethyl-C	1.16

Table 3. Significantly higher levels ($p < 0.05$ or (*) $p < 0.10$) of putatively identified DNA adducts of interest (relevant to the hypothesis) in precolonic meat digests (T0) after two sample differential analysis by means of Sieve™ database lookup.

Discriminating for	DNA adduct	RT (min)
Beef *vs.* Chicken	$1,N_6$-etheno-A	3.73
	Dimethyl-T or Ethyl-T	0.70
	Dimethyl-T or Ethyl-T	0.76
	Dimethyl-T or Ethyl-T	1.01
	Dimethyl-T or Ethyl-T	1.27
	Dimethyl-T or Ethyl-T	2.62
	Dimethyl-T or Ethyl-T	2.95
	Dimethyl-T or Ethyl-T	3.23
	Dimethyl-T or Ethyl-T	3.31
	Dimethyl-T or Ethyl-T	3.65
	Dimethyl-T or Ethyl-T	3.81
	Dimethyl-T or Ethyl-T (*)	4.37
	Ethyl-G or Dimethyl-G or Methoxymethyl-A	0.84
	M_2G (MDA dimer guanine DNA adduct)	1.14
	M_2G (MDA dimer guanine DNA adduct) (*)	3.73
	M_3C (Diformyldihydromethano-oxazocinyloxopyrimidine)	2.99
	Methoxymethyl-C	3.98
	Methoxymethyl-T	2.10
	Methyl-U	3.18
Chicken *vs.* Chicken + $CaCO_3$	Ethyl-G or Dimethyl-G or Methoxymethyl-A	0.85
	Hydroxy-A	3.16
	Methoxymethyl-C	3.64
	Methyl-C	2.68
	Methyl-C	2.98
	Methyl-C	3.24

Table 4. Significantly higher levels ($p < 0.05$ or $p < 0.10$ (*)) of putatively identified DNA adducts (not relevant to hypothesis) in pre-colonic meat digests (T0) after two sample differential analysis by means of Sieve™ database lookup.

Discriminating for	DNA adduct	RT (min)
Chicken *vs.* Beef	Carboxy-G (*)	0.81
	Heptenaletheno-G	4.01
	Hydroxy-A	3.22
	Hydroxy-A	3.48
	Hydroxy-A (*)	2.64
	Hydroxy-PhIP-G (Hydroxy-aminomethylphenylimidazopyridine-G) (*)	3.75
	Hydroxynonenal-A	2.65
	Hydroxynonenal-A	2.84
	IQ-G (Aminomethylimidazoquinolone-G)	1.29
	M_1A (MDA dimer guanine DNA adduct)	0.86
	M_1G (Pyrimidopurinone)	4.36
	M_3C (Diformyldihydromethano-oxazocinyloxopyrimidine)	1.05
	MeIQ-G (Aminodimethylimidazoquinolone-G)	5.08
	Methoxymethyl-C	0.77
	Methoxymethyl-T	3.62
	Methyl-A (*)	1.13
	Methyl-C	1.39
	Methyl-C	2.68
	Methyl-C (*)	3.80
	Methyl-T or Ethyl-U	3.16
	Methyl-T or Ethyl-U	3.84
	Methyl-T or Ethyl-U (*)	1.17
	PhIP-G (Aminomethylphenylimidazopyridine-G)	1.41
	ST-G (Sterigmatocystin-G)	1.72
	Tetramethyl-C	0.86
Chicken + $CaCO_3$ *vs.* Chicken	$1,N_6$-etheno-A	1.54
	Dimethyl-T or Ethyl-T	1.02
	Dimethyl-T or Ethyl-T	1.04
	Dimethyl-T or Ethyl-T	1.27
	Dimethyl-T or Ethyl-T	2.62
	Dimethyl-T or Ethyl-T	3.80
	Dimethyl-T or Ethyl-T	4.11
	Dimethyl-T or Ethyl-T	4.23
	Methoxymethyl-C	3.05
	Methoxymethyl-T	2.05
	Methyl-T or Ethyl-U	2.10
	Methyl-T or Ethyl-U	2.50
	Nitro-C	3.29
Beef + $CaCO_3$ *vs.* Beef	Dimethyl-T or Ethyl-T	0.71
	Dimethyl-T or Ethyl-T	1.02
	Dimethyl-T or Ethyl-T	1.28

Table 4 continued.

Discriminating for	DNA adduct	RT (min)
Beef + $CaCO_3$ *vs.* Beef	Dimethyl-T or Ethyl-T	1.62
	Dimethyl-T or Ethyl-T	2.87
	Dimethyl-T or Ethyl-T	2.70
	Dimethyl-T or Ethyl-T	4.22
	Hydroxynonenal-C	1.30
	Hydroxymethylhydantion (*)	2.80
	Diformyldihydromethano-oxazocinyloxopyrimidine **(**M_3C)	3.93
	Methoxymethyl-T	2.10
	Methyl-C (*)	2.89
	Methyl-T or Ethyl-U	2.51
	Methyl-U	2.42
	Methyl-U	3.90
	Nitro-C	2.76
	Nitro-C	3.29

3.3 UHPLC-MS/MS analysis of O^6-carboxymethylguanine and O^6-methylguanine in digests of different meat preparations

O^6-CMG could be detected upon digestion of all studied meat preparations, utilizing all fecal inocula obtained from both selected test subjects (P1 & P2), demonstrating a clear rise in O^6-CMG levels after 48 h of colonic digestion. The detected O^6-CMG levels at T0 did not surpass the limit of quantification, but the obtained O^6-CMG levels at T48 are shown in figure 4. O^6-MeG could not be detected in any of the samples.

Overall, the highest levels of the O^6-CMG DNA adduct were retrieved in digestive samples of test subject 1 (P1). Combining the results from all replicates (3 technical replicates x 3 biological replicates per test subject) from P1 demonstrated that O^6-CMG levels were significantly higher upon digestion of beef compared to chicken ($p < 0.05$), and that addition of $CaCO_3$ resulted in higher DNA adduct levels for both beef and chicken ($p < 0.05$). For test subject 2 (P2), the same results could be observed; O^6-CMG formation upon digestion of beef was significantly higher compared to chicken ($p < 0.05$), and addition of $CaCO_3$ did also result in a significant increase in O^6-CMG levels ($p < 0.05$). Combined statistical analysis of P1 and P2 data confirmed the earlier observed trend for beef vs. chicken across individuals; beef consumption increased O^6-CMG

levels overall ($p < 0.05$) (data not shown). This was not the case for $CaCO_3$ addition as it did not significantly affect O^6-CMG levels overall ($p > 0.05$) (data not presented).

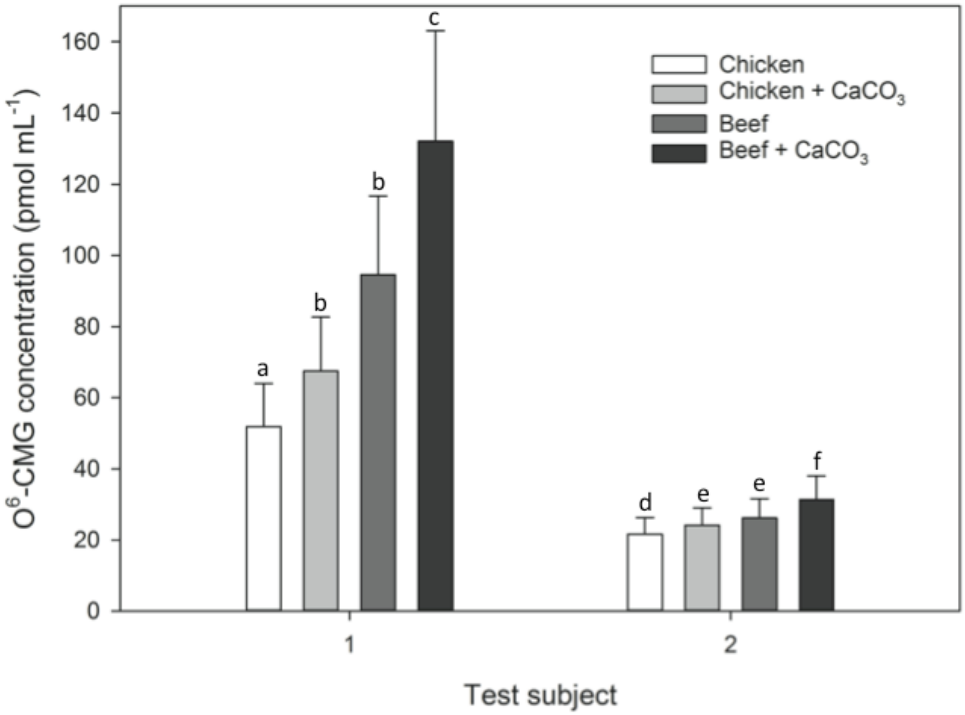


Figure 4. Formation of the O^6-CMG DNA adduct (mean concentration ± s.e.m.) during colonic digestion of chicken without added $CaCO_3$, chicken with added $CaCO_3$, beef without added $CaCO_3$ and beef with added $CaCO_3$ by means of the fecal inocula of test subject 1 and 2 (significant differences ($p < 0.05$) documented with different letters).

3.4 Malondialdehyde in digests of different meat preparations

The digestive samples were also subjected to MDA analysis. The formation of MDA upon digestion of beef was significantly higher compared to chicken individually and overall ($p < 0.01$). Addition of $CaCO_3$ to meat preparations rendered a significantly lower amount of MDA for P1 ($p < 0.05$), but not for P2 ($p > 0.05$) prior to colonic digestion (= in T0 samples). After colonic digestion, MDA levels in $CaCO_3$ meat digests were significantly reduced compared to digests of meat preparations without added $CaCO_3$ ($p < 0.05$) for both P1 and P2 (table 5 and 6).

Table 5. Malondialdehyde concentrations in P1 meat digests.

Meat type	Biological replicate	T0 MDA conc. (μ ± s.d.) (nmol/mL)		T48 MDA conc. (μ ± s.d.) (nmol/mL)	
		No added $CaCO_3$	Added $CaCO_3$	No added $CaCO_3$	Added $CaCO_3$
Chicken	1	29.17 ± 0.259	23.59 ± 0.133	25.4 ± 1.63	20.7 ± 2.43
	2	15.1 ± 1.88	14.1 ± 0.60	17.9 ± 1.11	16.1 ± 1.13
	3	27.2 ± 0.87	22 ± 3.1	25.0 ± 1.96	22.0 ± 0.41
	Mean	24 ± 13.5	20 ± 9.1	23 ± 8.7	20 ± 4.8
Beef	1	48.1 ± 2.23	32.4 ± 0.47	41.1 ± 1.60	27 ± 3.8
	2	17.7 ± 1.59	21.9 ± 2.16	17.8 ± 0.92	17.86 ± 0.071
	3	51.2 ± 2.77	45 ± 4.1	33 ± 3.7	37.4
	Mean	39 ± 16.9	33 ± 10.0	31 ± 10.0	24.5 ± 7.04

Table 6. Malondialdehyde concentrations in P2 meat digests.

Meat type	Biological replicate	T0 MDA conc. (μ ± s.d.) (nmol/mL)		T48 MDA conc. (μ ± s.d.) (nmol/mL)	
		No added $CaCO_3$	Added $CaCO_3$	No added $CaCO_3$	Added $CaCO_3$
Chicken	1	22.3 ± 0.87	16.7 ± 0.78	22.5 ± 1.33	15.6 ± 0.91
	2	28.6 ± 2.02	26.9 ± 0.75	17.2 ± 0.30	14.8 ± 0.58
	3	25.0 ± 1.34	24.1 ± 0.80	15.5 ± 0.70	15.5 ± 0.32
	Mean	25 ± 5.8	23 ± 6.8	18 ± 3.9	15.3 ± 2.47
Beef	1	22.8 ± 0.60	24 ± 3.6	26.3 ± 1.46	19.7 ± 1.26
	2	35 ± 3.9	36.62 ± 0.146	19.5 ± 1.23	18.7 ± 0.69
	3	29.6 ± 0.98	32.87 ± 0.283	18.41 ± 0.215	21.7 ± 0.89
	Mean	29 ± 5.1	31 ± 7.0	21 ± 4.4	20.1 ± 2.79

4. DISCUSSION

This study focused on the detection of diet-related DNA adducts in meat digests. Different putatively identified DNA adducts could be detected *in vitro* and several DNA adduct types were found to be more prevalent upon digestion of a particular meat type; e.g. levels of O^6-CMG and the putatively identified carboxyethyl-C were higher in beef vs. chicken digests, whilst both were also influenced by $CaCO_3$ supplementation. The latter also applies for the tentatively identified fapy-A and methoxymethyl-C (or its hydroxyethyl-C isomer) DNA adducts, which appeared to be discriminative for or significantly higher in meat that was not supplemented with $CaCO_3$. The lipid peroxidation product MDA increased upon digestion of beef compared to chicken, whereas $CaCO_3$ supplementation reduced its formation.

When comparing the concentrations of different food metabolites and DNA adduct levels in different meat type digests, the major compounds of interest in this study are the ones that are higher in beef compared to chicken since these types of molecules may help explain the link between red meat consumption and CRC incidence. Untargeted UHPLC-HRMS analysis revealed that the colonic microbiota of 3 out of 5 of the initial test subjects induced O^6-CMG formation upon digestion of meat. More in-depth targeted UHPLC-MS/MS analysis of P1 and P2 meat digest samples revealed the presence of the O^6-CMG DNA adduct in pre- and post-colonic digests with all 4 meat preparations. O^6-CMG levels were not quantifiable (< LOQ of 50 pmol per mg of DNA) prior to colonic digestion, but increased during the colonic digestion phase. The earlier reported finding that O^6-CMG levels were higher upon digestion of beef (heme-rich meat) compared to chicken (low heme content) [20] were confirmed in the present study. The red meat-CRC hypothesis suggests that the formation of NOCs in the gut is involved in the initiation and/or development of CRC. NOCs are known to have DNA-alkylating properties, rendering them to be a very likely precursor of O^6-CMG formation. This has already been investigated and demonstrated *in vivo* in humans as well, thus supporting the NOC hypothesis that links red meat consumption to CRC development [11].

An additional red meat-CRC hypothesis is the LPO hypothesis, in which LPOs are believed to stimulate initiation and/or promotion of CRC. In this study, MDA levels were higher in beef digests compared to chicken at all times, attesting to the pro-oxidative attributes of heme-rich meat [20, 22, 31]. *In vitro* MDA formation notably differed from O^6-CMG DNA adduct formation as MDA levels were higher after small bowel digestion of meat, demonstrating an

abiding decrease during colonic digestion. This decrease suggests active or passive degradation (through e.g. oxidation [32]) during colonic digestion although interaction with fibers [33], proteins [34] and/or DNA [8] is possible as well. However, the most prominent MDA-induced DNA adduct (M_1G) [8] could not be retrieved in this study. The interaction of MDA with proteins and/or fibers may thus be more prominent than DNA adduct formation although instability of the M_1G DNA adduct in digestive samples may also be the underlying cause of the lack of M_1G detection. Additional research should be conducted to support these statements.

To enable further investigation of the NOC and LPO hypothesis, meat preparations were supplemented with $CaCO_3$ since consumption of calcium-rich dairy products has been associated with a decreased CRC-risk [35, 36]. In this study however, addition of $CaCO_3$ stimulated O^6-CMG formation. It is however likely that calcium exerts its protective effect through a different route. After all, the CRC-protective attributes of calcium have been ascribed to its possible (concentration-dependent) anti-oxidative properties [5, 37, 38]. In support of this, we observed a decrease in MDA upon digestion of meat with added $CaCO_3$, distinctly suggesting that addition of $CaCO_3$ to meat reduces lipid peroxidation and MDA formation during digestion. This is an observation that has been documented before by Pierre et al. [39] who also measured TBARS levels after calcium supplementation to beef-fed rats. The finding that $CaCO_3$ addition stimulated O^6-CMG formation in this study may be linked to the composition and activity of the microbiota. In a previous study, we were able to demonstrate that O^6-CMG formation has a bacterial origin since inactivation of the microbiota completely abolished O^6-CMG production [20]. Stimulation of the bacteria that are responsible for O^6-CMG production may be the underlying cause of the $CaCO_3$ induced increase of O^6-CMG levels since varying dietary calcium levels can affect the microbial composition of the gut [40]. Some of the gut colonizing bacteria are able to perform enzymatic N-nitrosation reactions or reduce pH levels to induce acidic N-nitrosation, explaining the microbially induced formation of NOCs and alkylation DNA adducts [41, 42]. This could also explain the interfering role of $CaCO_3$. As such, future studies on red meat carcinogenicity should include characterization of the fecal microbiome to gather more knowledge on the possible involvement of specific gut microbes in the formation of (NOCs and) alkylation-induced DNA adducts. After all, previous research has already demonstrated that red meat and dietary fiber significantly alter O^6-MeG DNA adduct levels and the composition of the fecal microbiome [43].

Research on the composition of the human microbiome and metabolome has revealed a significant interindividual and intra-individual variability [44, 45]. Due to the highly variable and person-dependent bacterial composition in the colon and the resulting scope of metabolism activities, different metabolites – including DNA adducts or their precursors – can be present in the gut of different individuals at various points in time. Isolation of the diverse microbial community in the fecal inocula from different healthy volunteers could probably explain the notable interindividual variation in O^6-CMG levels and the obtained DNA adduct profile of P1 to P5 meat digests in this study. In pre-colonic digestion samples, DNA adducts can be present due to the active formation of genotoxic molecules during enzymatic (small intestinal) digestion of beef, followed by interaction with DNA (most likely from bacterial origin, as this type of DNA will be most abundantly present at the time). Alternatively, some DNA adducts may already be present in the pre-cultivated fecal inoculum [20]. Previous research demonstrated that the O^6-CMG DNA adduct (that could be detected in beef digests), and its unknown precursor, were not present after small bowel digestion but originated from the fecal inoculum of certain individuals. In addition, it was shown that the colonic microbiota significantly contributed to the formation of O^6-CMG during the simulated colonic digestion of both beef and chicken, demonstrating a clear rise of O^6-CMG in T48 samples [20]. However, these findings cannot be extrapolated for all other DNA adduct types and their respective precursors. Nevertheless, the presence of DNA adducts in pre-colonic meat digestion samples directs towards the presence of DNA-reactive molecules in the gut during or after digestion of food. In agreement, scientific literature suggests that (microbial) gut metabolism is not always beneficial to human health as it affects several physiological processes like inflammation, DNA damage and cell apoptosis. Especially some of the bacterially produced detrimental gut metabolites have potentially harmful alkylating and oxidant activities (e.g. NOCs and LPOs). Hence, evidence of the active contribution of these (microbially produced) gut metabolites like MDA and O^6-CMG to the development of chronic diseases is accumulating [15].

After UHPLC-HRMS DNA adductomics of all pre-colonic meat digests, ToxID™ analysis demonstrated that certain untargeted DNA adducts types were more prevalent in beef digests compared to digests of beef with added $CaCO_3$ or chicken digests. The same applies for chicken digests compared to digests of chicken without $CaCO_3$ for both test subjects. However, none of these ToxID™ retrieved DNA adduct types appeared to be significantly higher according to Sieve™ or Simca™. Simca™ analysis did however reveal the presence of a DNA adduct that

was discriminative for meat digests without added $CaCO_3$, and which was putatively identified as fapy-A, an oxidative DNA lesion with *in vivo* relevance [46]. Lower fapy-A levels in samples from $CaCO_3$ supplemented meat preparations could be due to the anti-oxidant nature of calcium in certain concentrations [37, 38], possibly explaining the CRC-protective characteristics of dairy (with high calcium levels) [36].

In post-colonic meat digests, Simca™ did not reveal any discriminant DNA adduct types, although both ToxID™ and Sieve™ did. One putatively identified DNA adduct type appeared to be significantly higher for beef compared to chicken in Sieve™ as well as ToxID™; i.e. carboxyethyl-C eluting at 0.82 min. Carboxyethyl-C is a DNA adduct type that can result from DNA alkylation, but has not been studied extensively or retrieved *in vivo* (to the best of our knowledge). Stimulation of the production of carboxyethyl-C during heme-rich meat digestion aligns with the earlier discussed results concerning the increased production of O^6-CMG upon digestion of beef, indicating a similar underlying pathway. Several potential isomers of methoxymethyl-C, also including hydroxyethyl-C, appeared to be higher in chicken digests without added $CaCO_3$ compared to chicken digests with supplemented $CaCO_3$. ToxID™ data analysis confirmed this for the methoxymethyl-C isomer eluting at 0.80 min. Since the formation of methoxymethyl-C or hydroxyethyl-C appears to be reduced upon $CaCO_3$ addition, this compound may be involved in the LPO hypothesis (just like MDA and fapy-A), although further identification and research are required to support this statement. To the best of our knowledge, both methoxymethyl-C and hydroxyethyl-C have not previously been detected *in vivo*.

Definite positive identification of the fapy-A, carboxyethyl-C and methoxymethyl-C (or hydroxyethyl-C) DNA adducts requires confirmation of co-chromatography by means of an analytical standard. Unfortunately, these 3 types of DNA adduct standards are not commercially available (at affordable rates), impairing confident compound identification. However, the fact that all compounds could be retrieved after specific sample preparation steps (DNA hydrolysis and DNA adduct extraction) and analytical analysis by means of an UHPLC-HRMS method that was developed for the detection of DNA adduct molecules specifically, makes it more likely that these compounds are indeed DNA adducts. In any case, all compounds that were determined to be higher in this study for one particular digested meat type can be defined as relevant food metabolites.

In this study, different ways of untargeted data processing were applied; i.e. ToxID™, Sieve™ and Simca™. However, Sieve™ and Simca™ did not indicate that O^6-CMG could be of any importance. Nonetheless, targeted O^6-CMG analysis and statistical interpretation of the results did reveal some interesting findings. This indicates that the obtained output of results very much depends on the applied software and parameter settings. In retrospect, we concluded that O^6-CMG signal intensities did not reach the minimal signal intensity that was set in Sieve™, excluding O^6-CMG from further multivariate data processing. However, this does not render the O^6-CMG data to be less important, keeping in mind that the initial untargeted detection of O^6-CMG was performed by means of an Orbitrap. An Orbitrap is very well suited for untargeted 'omics' applications, but is surpassed by triple quadrupoles regarding sensitivity [47]. In consequence, targeted UHPLC-MS/MS analysis by means of a triple quad enabled more in-depth investigation of O^6-CMG levels. This emphasizes that DNA adductomics applications can be very useful for preliminary biomarker screening, but also demonstrates the benefits of specialized data processing and/or complimentary analyses.

In this study, meat digestion was performed by means of an *in vitro* digestion system that offers several advantages due to its flexibility and lack of ethical restraints [19]. Unfortunately, its use is quite labor-intensive, which is reflected in the fact that the microbiota of only 5 different individuals could be screened and that only 2 were selected for a more in-depth investigation of meat-related DNA adduct formation. In addition, the *in vitro* setup may not truly reflect the *in vivo* situation since the *in vitro* system mainly mimics luminal DNA adduct formation, which may not translate directly into luminal and mucosal DNA adduct formation *in vivo*. Future *in vitro* studies in line with this study, and future *in vivo* studies in humans should document dietary habits (e.g. make use of food frequency questionnaires, 24-hour recalls, etc.) to be able to link this information on exposure to the variation in detected DNA adduct levels. Furthermore, complimentary analyses to measure the actual intake of nutrients, as well as assessment of non-digested nutrients and digestion metabolites (e.g. with fecal metabolomics [48]) may offer some relevant information to help elucidate the red meat-CRC link.

5. CONCLUSION

This study was able to confirm that heme-rich meat stimulates lipid peroxidation and O^6-CMG formation. It also demonstrated that $CaCO_3$ can suppress MDA formation, acknowledging the CRC-protective potential of calcium ingestion via the reduction of lipid peroxidation. In contrast, $CaCO_3$ appeared to stimulate alkylation of guanine (O^6-CMG formation), which is believed to be procarcinogenic [11]. Digestion of different meat types revealed that both meat digestion and $CaCO_3$ supplementation influences the DNA adductome. The presence of the fapy-A, carboxyethyl-C and methoxymethyl-C DNA adducts in meat digests does however require further investigation. Since all putatively identified DNA adduct types in this meat digestion study originate from DNA alkylation, DNA oxidation or attack of DNA nucleobases by LPOs [25], further DNA adduct profiling and identification is definitely warranted to allow thorough investigation of the LPO and NOC pathway in red meat related carcinogenicity. This will be enabled by future *in vitro* and *in vivo* DNA adductome studies. To the best of our knowledge, this is one of very few studies to implement a state-of-the-art DNA adductomics platform. Moreover, to date, it is the only study that employed this approach to investigate DNA adduct formation during food digestion.

REFERENCES

[1] J. Ferlay, I. Soerjomataram, M. Ervik, R. Dikshit, S. Eser, C. Mather, M. Rebelo, D.M. Parkin, D. Forman, F. Bray, GLOBOCAN 2012 v1.0, Cancer Incidence and Mortality Worldwide, GLOBOCAN 2012; International Agency for Research on Cancer. Available from: http://globocan.iarc.fr, (2013).
[2] P. Anand, A.B. Kunnumakara, C. Sundaram, K.B. Harikumar, S.T. Tharakan, O.S. Lai, B. Sung, B.B. Aggarwal, Cancer is a Preventable Disease that Requires Major Lifestyle Changes, Pharmaceutical Research, 25 (2008) 2097-2116.
[3] L.A. Torre, F. Bray, R.L. Siegel, J. Ferlay, J. Lortet-Tieulent, A. Jemal, Global cancer statistics, 2012, CA: a cancer journal for clinicians, 65 (2015) 87-108.
[4] D.E. Corpet, Red meat and colon cancer: should we become vegetarians, or can we make meat safer?, Meat science, 89 (2011) 310-316.
[5] M. Oostindjer, J. Alexander, G.V. Amdam, G. Andersen, N.S. Bryan, D. Chen, D.E. Corpet, S. De Smet, L.O. Dragsted, A. Haug, A.H. Karlsson, G. Kleter, T.M. de Kok, B. Kulseng, A.L. Milkowski, R.J. Martin, A.M. Pajari, J.E. Paulsen, J. Pickova, K. Rudi, M. Sodring, D.L. Weed, B. Egelandsdal, The role of red and processed meat in colorectal cancer development: a perspective, Meat science, 97 (2014) 583-596.

[6] U. Nair, H. Bartsch, J. Nair, Lipid peroxidation-induced DNA damage in cancer-prone inflammatory diseases: A review of published adduct types and levels in humans, Free Radical Biology and Medicine, 43 (2007) 1109-1120.
[7] L.J. Marnett, Oxyradicals and DNA damage, Carcinogenesis, 21 (2000) 361-370.
[8] L.J. Marnett, Lipid peroxidation-DNA damage by malondialdehyde, Mutation research, 424 (1999) 83-95.
[9] T.B. Kryston, A.B. Georgiev, P. Pissis, A.G. Georgakilas, Role of oxidative stress and DNA damage in human carcinogenesis, Mutation research, 711 (2011) 193-201.
[10] D.E.G. Shuker, G.P. Margison, Nitrosated Glycine Derivatives as a Potential Source of O6-Methylguanine in DNA, Cancer Research, 57 (1997) 366-369.
[11] M.H. Lewin, N. Bailey, T. Bandaletova, R. Bowman, A.J. Cross, J. Pollock, D.E.G. Shuker, S.A. Bingham, Red Meat Enhances the Colonic Formation of the DNA Adduct O6-Carboxymethyl Guanine: Implications for Colorectal Cancer Risk, Cancer Research, 66 (2006) 1859-1865.
[12] W. Lijinsky, N-Nitroso compounds in the diet, Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 443 (1999) 129-138.
[13] P.C. Dedon, S.R. Tannenbaum, Reactive nitrogen species in the chemical biology of inflammation, Archives of Biochemistry and Biophysics, 423 (2004) 12-22.
[14] W. Lijinsky, Chemistry and biology of N-nitroso compounds, Cambridge Monographs on Cancer Research, (1992) 480.
[15] P. Louis, G.L. Hold, H.J. Flint, The gut microbiota, bacterial metabolites and colorectal cancer, Nat Rev Micro, 12 (2014) 661-672.
[16] L.Y. Hemeryck, L. Vanhaecke, Diet-related DNA adduct formation in relation to carcinogenesis, Nutr Rev, 74 (2016) 475-489.
[17] M.C. Poirier, Chemical-induced DNA damage and human cancer risk, Nat Rev Cancer, 4 (2004) 630-637.
[18] R. Colin Garner, The role of DNA adducts in chemical carcinogenesis, Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 402 (1998) 67-75.
[19] T. Van Hecke, J. Vanden Bussche, L. Vanhaecke, E. Vossen, J. Van Camp, S. De Smet, Nitrite curing of chicken, pork, and beef inhibits oxidation but does not affect N-nitroso compound (NOC)-specific DNA adduct formation during in vitro digestion, J Agric Food Chem, 62 (2014) 1980-1988.
[20] J. Vanden Bussche, L.Y. Hemeryck, T. Van Hecke, G.G. Kuhnle, F. Pasmans, S.A. Moore, T. Van de Wiele, S. De Smet, L. Vanhaecke, O(6)-carboxymethylguanine DNA adduct formation and lipid peroxidation upon in vitro gastrointestinal digestion of haem-rich meat, Molecular nutrition & food research, 58 (2014) 1883-1896.
[21] T. Van Hecke, E. Vossen, L.Y. Hemeryck, J. Vanden Bussche, L. Vanhaecke, S. De Smet, Increased oxidative and nitrosative reactions during digestion could contribute to the association between well-done red meat consumption and colorectal cancer, Food chemistry, 187 (2015) 29-36.
[22] T. Van Hecke, E. Vossen, J. Vanden Bussche, K. Raes, L. Vanhaecke, S. De Smet, Fat content and nitrite-curing influence the formation of oxidation products and NOC-specific DNA adducts during in vitro digestion of meat, PloS one, 9 (2014) e101122.
[23] J. Winter, L. Nyskohus, G.P. Young, Y. Hu, M.A. Conlon, A.R. Bird, D.L. Topping, R.K. Le Leu, Inhibition by resistant starch of red meat-induced promutagenic adducts in mouse colon, Cancer prevention research (Philadelphia, Pa.), 4 (2011) 1920-1928.
[24] J. Winter, G.P. Young, Y. Hu, S.W. Gratz, M.A. Conlon, R.K. Le Leu, Accumulation of promutagenic DNA adducts in the mouse distal colon after consumption of heme does not

induce colonic neoplasms in the western diet model of spontaneous colorectal cancer, Molecular nutrition & food research, 58 (2014) 550-558.
[25] L.Y. Hemeryck, A.I. Decloedt, J. Vanden Bussche, K.P. Geboes, L. Vanhaecke, High resolution mass spectrometry based profiling of diet-related deoxyribonucleic acid adducts, Anal Chim Acta, 892 (2015) 123-131.
[26] K. Molly, M.V. Woestyne, I.D. Smet, W. Verstraete, Validation of the Simulator of the Human Intestinal Microbial Ecosystem (SHIME) Reactor Using Microorganism-associated Activities, Microbial Ecology in Health and Disease, 7 (1994) 191-200.
[27] J. Vanden Bussche, S.A. Moore, F. Pasmans, G.G. Kuhnle, L. Vanhaecke, An approach based on ultra-high pressure liquid chromatography-tandem mass spectrometry to quantify O6-methyl and O6-carboxymethylguanine DNA adducts in intestinal cell lines, Journal of chromatography. A, 1257 (2012) 25-33.
[28] L. Van Meulebroek, A Metabolomic Framework to Study the Regulating Role of Phytohormones Towards Carotenoids in Tomato Fruit, PhD Thesis, Ghent University, Merelbeke, Belgium, (2014).
[29] M.N. Triba, L. Le Moyec, R. Amathieu, C. Goossens, N. Bouchemal, P. Nahon, D.N. Rutledge, P. Savarin, PLS/OPLS models in metabolomics: the impact of permutation of dataset rows on the K-fold cross-validation quality parameters, Molecular bioSystems, 11 (2015) 13-19.
[30] D. Grotto, L.D. Santa Maria, S. Boeira, J. Valentini, M.F. Charão, A.M. Moro, P.C. Nascimento, V.J. Pomblum, S.C. Garcia, Rapid quantification of malondialdehyde in plasma by high performance liquid chromatography–visible detection, Journal of Pharmaceutical and Biomedical Analysis, 43 (2007) 619-624.
[31] F. Pierre, A. Freeman, S. Tache, R. Van der Meer, D.E. Corpet, Beef meat and blood sausage promote the formation of azoxymethane-induced mucin-depleted foci and aberrant crypt foci in rat colons, The Journal of nutrition, 134 (2004) 2711-2716.
[32] T. Nosova, K. Jokelainen, P. Kaihovaara, H. Jousimies-Somer, A. Siitonen, R. Heine, M. Salaspuro, Aldehyde dehydrogenase activity and acetate production by aerobic bacteria representing the normal flora of human large intestine, Alcohol Alcohol, 31 (1996) 555-564.
[33] S.J. Hur, B.O. Lim, G.B. Park, S.T. Joo, Effects of Various Fiber Additions on Lipid Digestion during In Vitro Digestion of Beef Patties, Journal of Food Science, 74 (2009) C653-C657.
[34] K. Houglum, M. Filip, J.L. Witztum, M. Chojkier, Malondialdehyde and 4-hydroxynonenal protein adducts in plasma and liver of rats with iron overload, J Clin Invest, 86 (1990) 1991-1998.
[35] J.A. Baron, M. Beach, J.S. Mandel, R.U. van Stolk, R.W. Haile, R.S. Sandler, R. Rothstein, R.W. Summers, D.C. Snover, G.J. Beck, J.H. Bond, E.R. Greenberg, Calcium supplements for the prevention of colorectal adenomas. Calcium Polyp Prevention Study Group, N Engl J Med, 340 (1999) 101-107.
[36] M. Huncharek, J. Muscat, B. Kupelnick, Colorectal cancer risk and dietary intake of calcium, vitamin D, and dairy products: a meta-analysis of 26,335 cases from 60 observational studies, Nutrition and cancer, 61 (2009) 47-69.
[37] M.A. Babizhayev, The biphasic effect of calcium on lipid peroxidation, Arch Biochem Biophys, 266 (1988) 446-451.
[38] W. Bors, G.R. Buettner, C. Michel, M. Saran, Calcium in lipid peroxidation: does calcium interact with superoxide?, Arch Biochem Biophys, 278 (1990) 269-272.
[39] F. Pierre, R. Santarelli, S. Tache, F. Gueraud, D.E. Corpet, Beef meat promotion of dimethylhydrazine-induced colorectal carcinogenesis biomarkers is suppressed by dietary calcium, Br J Nutr, 99 (2008) 1000-1006.

[40] B.U. Metzler-Zebeli, W. Vahjen, T. Baumgartel, M. Rodehutscord, R. Mosenthin, Ileal microbiota of growing pigs fed different dietary calcium phosphate levels and phytase content and subjected to ileal pectin infusion, J Anim Sci, 88 (2010) 147-158.
[41] A. Engemann, C. Focke, H.U. Humpf, Intestinal formation of N-nitroso compounds in the pig cecum model, J Agric Food Chem, 61 (2013) 998-1005.
[42] T. Sobko, C.I. Reinders, E. Jansson, E. Norin, T. Midtvedt, J.O. Lundberg, Gastrointestinal bacteria generate nitric oxide from nitrate and nitrite, Nitric Oxide, 13 (2005) 272-278.
[43] R.K. Le Leu, J.M. Winter, C.T. Christophersen, G.P. Young, K.J. Humphreys, Y. Hu, S.W. Gratz, R.B. Miller, D.L. Topping, A.R. Bird, M.A. Conlon, Butyrylated starch intake can prevent red meat-induced O6-methyl-2-deoxyguanosine adducts in human rectal tissue: a randomised clinical trial, Br J Nutr, 114 (2015) 220-230.
[44] P. Bernini, I. Bertini, C. Luchinat, S. Nepi, E. Saccenti, H. Schafer, B. Schutz, M. Spraul, L. Tenori, Individual human phenotypes in metabolic space and time, J Proteome Res, 8 (2009) 4264-4271.
[45] P.J. Turnbaugh, R.E. Ley, M. Hamady, C. Fraser-Liggett, R. Knight, J.I. Gordon, The human microbiome project: exploring the microbial part of ourselves in a changing world, Nature, 449 (2007) 804-810.
[46] R. De Bont, N. van Larebeke, Endogenous DNA damage in humans: a review of quantitative data, Mutagenesis, 19 (2004) 169-185.
[47] N. Tretyakova, P.W. Villalta, S. Kotapati, Mass Spectrometry of Structurally Modified DNA, Chemical Reviews, 113 (2013) 2395-2436.
[48] J. Vanden Bussche, M. Marzorati, D. Laukens, L. Vanhaecke, Validated High Resolution Mass Spectrometry-Based Approach for Metabolomic Fingerprinting of the Human Gut Phenotype, Anal Chem, 87 (2015) 10927-10934.

CHAPTER V

Shifts in the *in vitro* DNA adductome due to red *vs.* white meat digestion

Adapted from:

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ABSTRACT

In 2015, the WHO and IARC issued that the consumption of red meat is 'probably carcinogenic to humans', primarily focusing on the observed link between red meat consumption, as a part of the Western diet, and CRC incidence. Different hypotheses have been put forward to explain this causal relationship but the heme hypothesis, which states that heme iron present in red meat stimulates the formation of toxic NOCs and LPOs, has received the most attention and support. Although we know that NOCs as well as LPOs can exert DNA damaging effects, the exact underling mechanisms of red meat and heme iron genotoxicity requires further elucidation. By means of DNA adductomics, chemically induced DNA adduct formation can be mapped in relation to e.g. dietary exposures. In this study, this state-of-the-art methodology was used to investigate alkylation and (lipid per)oxidation induced DNA adduct formation in *in vitro* red *vs.* white meat digests. In doing so, 90 DNA adduct types could be (tentatively) identified, encompassing several known alkylation and (lipid per)oxidation induced DNA adducts. The retrieved DNA adduct types and levels demonstrate a clear interindividual variation. Overall, the increased formation of 26 DNA adduct types could be related to red meat (and heme iron) digestion (as opposed to white meat digestion). More specifically, hydroxymethylhydantion, diformyldihydromethano-oxazocinyloxopyrimidine (M_3C), hydroxyethylthymine (or methoxymethylthymine), carboxyethylthymine, methylthymine, ethylthymine, hydroxymethylthymine, tetramethylthymine and 3,N^4-ethenocytosine were singled out as potential heme-rich meat digestion markers. Since those particular DNA adduct types originate from DNA alkylation and/or oxidation processes, this is in support of the heme, NOC and LPO hypothesis. As a result, it has become clear that DNA adduct formation may indeed contribute to red meat related CRC risk, arguing further investigation.

Keywords:

Cancer Risk, DNA Adductome Mapping, Heme, Red Meat

1. INTRODUCTION

The vast majority of cancer cases are not hereditary in origin, but are caused by the (chronic) exposure to certain environmental factors. This encompasses exposure to genotoxic chemicals from multiple and highly diverse sources; e.g. HCAs in meat cooked at high temperatures, mycotoxins in molded food and feed, PAHs in tobacco smoke, diesel exhaust, and grilled meat [1]. Such chemicals can contribute to cancer initiation and development individually and/or synergistically. Moreover, the hence induced DNA adduct formation appears to be key in chemically induced carcinogenesis; covalent binding of genotoxic chemicals to DNA nucleobases can alter genes and induce mutations [2].

In 2015, the IARC and the WHO issued that red meat is 'probably carcinogenic to humans' (group 2B) "based on limited evidence that the consumption of red meat causes cancer in humans and strong mechanistic evidence supporting a carcinogenic effect" [3]. One of the main hypothetical mechanisms underlying the epidemiological link between red meat consumption and CRC is explained by the 'heme hypothesis'. This hypothesis states that heme, which is intrinsically more present in red meat compared to white meat, stimulates (lipid per)oxidation and NOC formation in the gut besides effecting direct toxicity [4, 5]. NOCs, heme, as well as several known oxidative metabolites (e.g. ROS and LPOs) exert genotoxic effects *via* alkylation and/or oxidation of DNA, as such contributing to chemically induced DNA adduct formation [6-8].

NOC exposure can occur *via* different routes; dietary intake and endogenous formation in the gut [9, 10]. More specifically, nitrosamines and nitrosamides can be formed in the stomach due to the interaction of nitric oxide or nitrite from metabolism, food, saliva and pharmaceutical drugs [10, 11]. In the large bowel, microbial fermentation of proteins can lead to the production of amines, which can then be transformed to NOCs by means of nitrosation [12]. Several studies have demonstrated that dietary heme iron, but not inorganic iron and/or meat protein, significantly increases fecal NOC-levels of human volunteers consuming a red meat diet [13-16]. Hence, it appears that heme iron catalyzes NOC-formation upon red meat digestion. More specifically, it has been hypothesized that heme can capture NO (e.g. after release by S-nitrosothiols under alkaline conditions in the small bowel), resulting in the formation of nitrosyl heme. Thus, since nitrosyl heme can act as a nitrosating agent, heme iron can promote

endogenous NOC formation [17]. Because NOCs exert DNA-alkylating properties, increased NOC formation can lead to the accumulation of alkylation-induced DNA adducts [6, 18].

The heme hypothesis also stipulates a direct and indirect heme iron induced increase of oxidative stress and lipid peroxidation. Through the Fenton reaction, heme iron can stimulate the formation of ROS and LPOs [19], leading to a cascade of oxidative reactions and resulting in the oxidation of e.g. DNA nucleobases. As such, red meat digestion can increase CRC risk in a twofold manner; i.e. through the induction of oxidative stress and/or by DNA adduct formation [4, 5, 20, 21].

DNA adduct formation due to red meat consumption could be an important step in the pathophysiology underlying CRC. However, to date, the exact etiology of red meat induced CRC initiation, promotion and progression lacks full elucidation. This study aimed to further unravel the genotoxic effects of red meat consumption *via* alkylation and/or oxidation induced DNA adduct formation. To this purpose, gastrointestinal digestion of beef diaphragm (a model for red meat) was simulated *in vitro*, and compared to the digestion of chicken breast (a model for white meat). Analysis of any resulting DNA adduct formation was performed by means of a state-of-the-art DNA adductomics platform based on the use of high resolution mass spectrometry, and an in-house DNA adduct database listing all currently known diet-related alkylation and (lipid per)oxidation related DNA adducts [6, 22]. In addition, to gain a more profound insight into the underlying mechanisms, additional experiments were performed to assess the possible interfering role of myoglobin, i.e. the heme iron containing protein that is intrinsically more present in red compared to white meat [20].

2. MATERIALS AND METHODS

2.1 Reagents and chemicals

O^6-CMdG was kindly provided by Dr. S. Moore from Liverpool John Moores University (UK). Deoxyguanosine (dG), O^6-MedG and O^6-d_3-MedG (internal standard for both O^6-MedG and O^6-CMdG) were purchased from Sigma-Aldrich (St. Louis, USA). Analytical standards for M_1G

and its internal standard M_1G-$^{13}C_3$ were obtained from Toronto Research Chemicals (Toronto, Canada).

O^6-CMdG, O^6-MedG, O^6-d_3-MedG, and dG were hydrolyzed to their nucleobase form in 0.1 M formic acid over the course of 30 min at 80°C. All standards were diluted in methanol to obtain stock and working solutions of 500 ng/mL and 5 ng/mL, respectively.

Myoglobin was obtained from Sigma-Aldrich (St-Louis, Missouri, USA). A stock solution of 10 mg/mL for myoglobin was prepared in ultrapure water (UP) (Millipore, Brussels, Belgium) and stored at -20°C.

Solvents were of analytical grade (VWR International, Merck, Darmstadt, Germany) when used for extraction and purification steps, and of Optima LC/MS grade for LC−MS application (Fisher Scientific UK, Loughborough, UK).

2.2 Meat preparations

Beef diaphragm, chicken breast and subcutaneous pork fat (lard) were obtained from a local slaughterhouse and butcher. The beef and chicken meat were chopped into cubes (1 to 2 cm^3), after which lard was added to obtain a total fat content of 20 %. The meat preparations were minced (with an Omega T-12 (Omega Foodtech, Bologna, Italy) equipped with a 10-mm plate) and ground (with a 3.5-mm plate) thoroughly. Subsequently, the meat preparations were heated in a hot water bath (GFL, Grossburgwedel, Germany) for 30 min after reaching a core temperature of 90°C. As a final step, the meat preparations were homogenized with a food processor, after which they were stored at -20°C.

2.3 *In vitro* gastrointestinal digestion of meat preparations

2.3.1 Collection, storage and pre-cultivation of colonic microbiota

Fresh fecal samples were obtained from 7 male and 3 female volunteers (age ranging from 22 to 75 yrs. old) without any medical history of gastrointestinal disease. None of the solicited volunteers underwent antibiotic treatment during at least 6 months prior to donation. All 10

human donors of fecal material were recruited among the laboratory personnel and their family members through informal announcement, after which all participating volunteers gave their written informed consent. The obtained data and volunteer information were analyzed anonymously and de-identified. The research was approved by the Federal Public Service of Health, Food Chain Safety and Environment, Belgium, but there was no need to submit an application to the ethical committee due to the non-invasive nature of the voluntary donation of fecal samples.

Fresh fecal samples were processed according to a protocol adapted from Molly *et al.* [23, 24] as has been described previously [25]. In short, fresh fecal material was diluted in preheated PBS solution (1:4; w/v) to which sodium thioglycolate (1 g/L) was added as a reducing agent. Subsequently, the fecal slurry was filtered (through a 1 mm metal sieve) and stored at -80°C on a glycerol stock (20 %). Prior to the gastrointestinal digestion of meat, the fecal inocula was pre-cultivated anaerobically for 24 h at 37°C in BHI broth (obtained from Oxoid Ltd, Hampshire, GB) with added cysteine (37 g/L BHI + 0.5 g/L cysteine) at a 1:9 ratio (v/v)).

2.3.2 Simulated gastrointestinal digestion of meat preparations

The use of a well-established *in vitro* gastrointestinal digestion model enabled simulation of stomach, small and large bowel digestion of beef and chicken meat preparations. The utilized model has been described on multiple occasions. Therefore, for all details on the utilized *in vitro* digestion model and the prior preparation of all mimicked gastrointestinal juices, brain heart infusion broth and SHIME medium, we refer to previous work [25].

For this study, 4.5 g of beef or chicken meat preparations were digested *in vitro* in triplicate, using 10 different fecal inocula (n = 2 x 3 x 10 simulated meat digestions). Samples were taken after simulation of duodenal digestion ("T0" samples, whereby sampling took place immediately after addition of SHIME medium and the fecal inoculum; i.e. just prior to colonic fermentation) and at the end of the simulated colonic meat fermentation ("T48" samples, whereby sampling took place after 48 h incubation with SHIME medium and fecal inocula; i.e. after the colonic fermentation). All meat digestion samples were stored at -80°C until analysis.

To investigate the role of heme iron in red meat induced genotoxicity, an additional experiment involving myoglobin addition was performed. The following digestions were performed in triplicate; 4.5 g of beef meat preparation (produced as described previously; 2.2 Meat preparations) without added myoglobin, 4.5 g of beef meat preparation with 5 mg of added myoglobin, 4.5 g of beef meat preparation with 50 mg of added myoglobin, 50 mg of myoglobin (without meat or lard), and 4.5 g of lard with 50 mg of added myoglobin. Samples were obtained at "T0" and "T48" and stored at -80°C. The fecal inoculum used to perform the colonic fermentation was selected at random (P5).

2.4 DNA adductomics analysis

2.4.1 Sample preparation

DNA adducts in meat digests were extracted and purified according to a protocol described by Vanden Bussche *et al.* [26] and Hemeryck *et al.* [22]. At first, 3 internal standards (O^6-d_3-MeG and M_1G-$^{13}C_3$) were added to each sample. Then, DNA was hydrolyzed in 0.1 M formic acid in UP (30 min, 80°C) to cleave both adducted and non-adducted DNA nucleobases from all DNA sequences present in the meat digestion samples. Subsequently, sample purification and cleanup was performed by means of SPE (Oasis® HLB cartridges (1 cc, 30 mg) Waters (Milford, USA)), after which the eluted samples were evaporated to dryness (90 min under vacuum, 20°C). Finally, the dried residue was re-suspended in 100 µL of 0.05 % of acetic acid in UP and stored at -20°C awaiting analysis.

2.4.2 UHPLC-HRMS analysis

Analysis of DNA adducts in meat digests was enabled by ultrahigh performance liquid chromatography coupled to high resolution mass spectrometry (UHPLC-HRMS) [22]. In brief, chromatographic separation was performed with an Acquity BEH C18 Waters column (1.7 µm, 2.1 x 100 mm; Waters Corporation, Milford, USA). The mobile phases consisted of 0.05 % of acetic acid in UP and 100 % methanol. The flow of the mobile phases (300 µL per min) and injection of samples was accomplished with a Dionex Ultimate 3000 pump and autosampler

(Thermo Scientific, San José, USA), and HRMS DNA adduct analysis was performed by means of a hybrid Quadrupole-Orbitrap High Resolution Accurate Mass Spectrometer (HRAM, Q-Exactive, Thermo Fisher Scientific, San José, USA) coupled to a heated electrospray ionization (HESI-II) source. Internal calibration of the MS system was performed daily by infusion of calibration mixtures that were prepared according to the protocol described in the operations manual (Thermo Fisher Scientific, San José, USA). General instrument control and initial data processing were performed with Chromeleon Xpress and Xcalibur™ 3.0.

2.4.3 Data processing and statistics

2.4.3.1 ToxFinder™ profiling

The use of ToxFinder™ 1.0 software (Thermo Fisher Scientific, San José, USA) and an in-house DNA adduct database allowed screening of the full scan HRMS spectra of meat digestion samples for alkylation and/or (per)oxidation induced DNA adducts. Only DNA adducts demonstrating a minimum signal intensity of 20,000, a maximum mass deviation of 10 ppm, recurrence and stable RT in replicate samples, and the presence of the naturally occuring C^{13} isotope were retained. The hence obtained output was visualized by means of Morpheus software (https://software.broadinstitute.org/morpheus). Student's t-test was used for statistical interpretation of the detected DNA adduct levels. Tentative identification based on accurate mass was checked manually for each compound. The identities of O^6-MeG, O^6-O^6-CMG and M_1G were confirmed by means of analytical standards.

2.4.3.2 Sieve™ pre-processing

To screen digested meat samples for known alkylation and/or oxidation induced DNA adducts, control compare trend analysis was performed using the database lookup function of Sieve™ 2.2 (Thermo Fisher Scientific, San José, USA). Combining the spectral data obtained from the three technical replicate digestion samples assured repeatability of the obtained results. Only ions with an m/z between 70 and 700 Da, and eluting between 0.7 and 5.6 min of chromatographic analysis were considered. A mass deviation up to 10 ppm was allowed, whilst the maximum peak width consisted of 0.5 min. Chromatographic peak selection was executed for positive and

negative ions separately. The maximum number of frames and minimal peak intensity were set at 200,000 and 20,000 arbitrary units, respectively. After automated processing of all raw files, the database lookup function was enabled to match the retrieved matrix features to DNA adduct identities listed in an in-house diet-related DNA adduct database, enabling tentative DNA adduct identification.

2.4.3.3 Simca™ multivariate statistics

Simca™ 14 software (Umetrics AB, Umeå, Sweden) was used for multivariate statistics, starting with the importation of the output of Sieve™ pre-processing. Overall, data analysis was performed combining the data of all 3 technical replicates at all times to ensure robustness and repeatability.

At first, Principal Component Analysis (PCA) was performed to enable preliminary data exploration and detection of possible outliers. Subsequently, logarithmic data transformation and Pareto scaling were performed, followed by automated OPLS-DA data modelling. The validity of the obtained OPLS-DA model was checked with permutation testing (n = 100), CV-ANOVA ($p < 0.05$), and assessment of R^2 (must approach 1 (= perfect fit)) and Q^2 (=cross-validated R^2, operated with a minimal threshold of 0.5 to ensure correct prediction). For valid models, discriminative ions were selected based on a VIP score > 0.8 because a VIP > 1 demonstrates a high influence, a VIP > 0.8 (but < 1) demonstrates a moderate influence, but a compound with a VIP < 1 merely demonstrates a low influence [27, 28].

3. RESULTS

3.1 DNA adduct profiling of red *vs.* white meat digests

Chicken and beef digests (T0 as well as T48 samples) were screened for the presence of alkylation and/or oxidation induced DNA adducts by means of ToxFinder™ and the in-house DNA adduct database, enabling comparison of the levels of the retrieved DNA adduct types in different sample types.

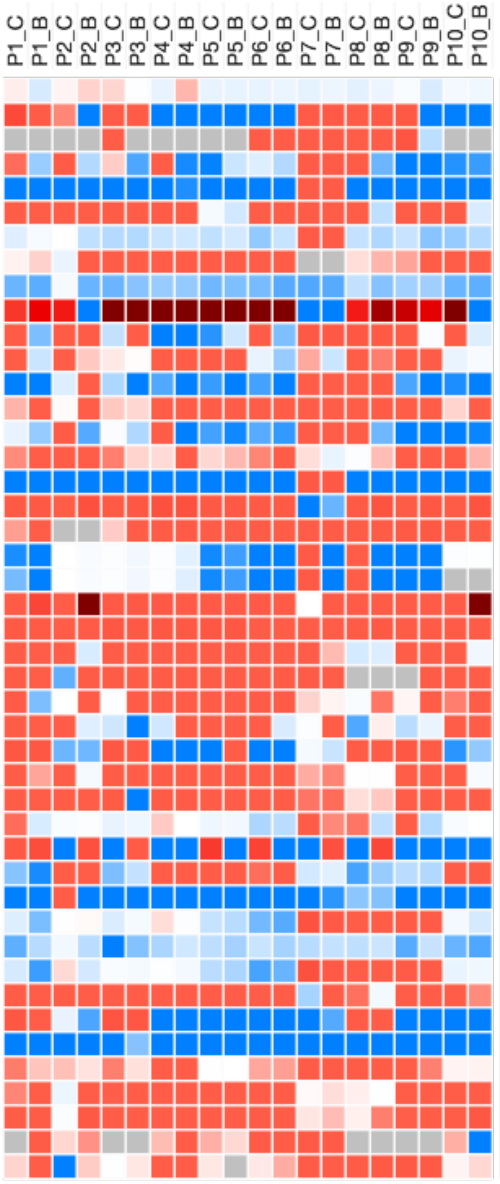
3.1.1 Formation or degradation of DNA adducts during colonic fermentation

The detected concentrations of some DNA adduct types increased during colonic fermentation, whilst others declined. In figure 1, the levels of several tentatively identified DNA adduct types in T48 samples are compared to those in T0 samples by means of a heat map. Comparison was enabled by subtracting T0 DNA adduct levels from T48 DNA adduct levels (peak areas) after correction for individual sample guanine content, and displaying the difference by means of a color scheme; higher T48 levels (compared to T0) are displayed in red (the result of the T48 - T0 subtraction is positive), whilst lower T48 levels (compared to T0) are displayed in blue (the result of the T48 - T0 subtraction is negative). The heat map in figure 1 displays significant as well as non-significant differences. In figure 2, only significant differences are shown.

3.1.2 The effect of beef digestion in comparison to chicken digestion

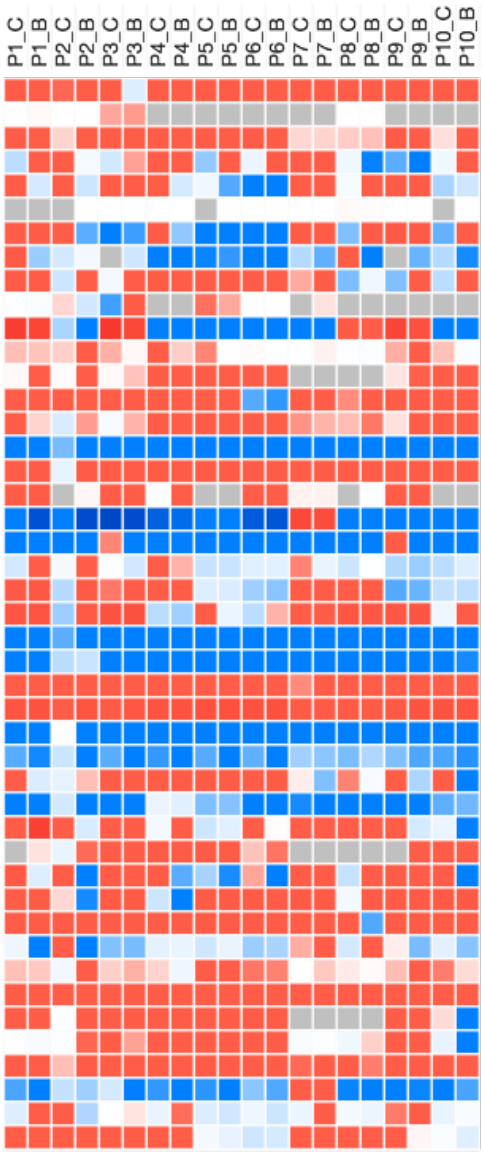
3.1.2.1 At the start of the colonic fermentation (T0)

In figure 3, the difference in DNA adduct levels in T0 beef digests is compared to T0 chicken digests. To enable straightforward comparison, measured DNA adduct levels (peak areas corrected for the amount of guanine in each sample) in chicken digests were subtracted from those in beef digests. When DNA adduct levels in beef digests were higher in comparison to chicken digests, this is displayed in red, whilst when DNA adduct levels in chicken digests were higher than those in beef digests, this is displayed in blue. The heat map in figure 3 displays significant as well as non-significant differences. Figure 4 only shows significant differences.



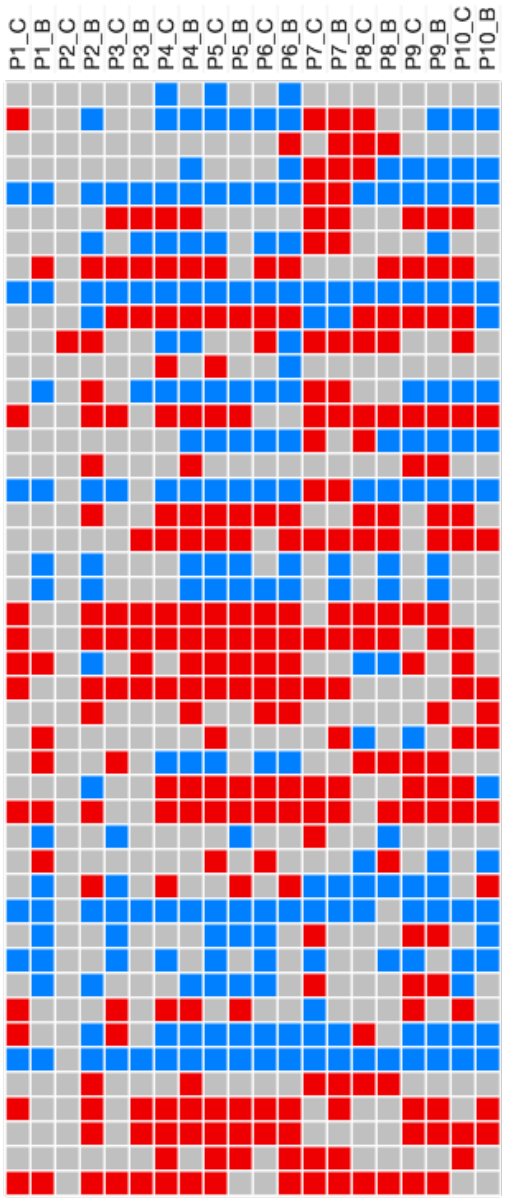
Compound	RT
Carbamoylhydroxyethyl-G (+)	0.81
Hydroxyethyl-C or Methoxymethyl-C (+)	0.89
Dimethyl-C or Ethyl-C (+)	0.98
Hydroxyethyl-T or Methoxymethyl-T (+)	1.01
Trihydroxybutyl-U (-)	1.02
Trihydroxybutyl-T (-)	1.04
Carboxyethyl-T (+)	1.06
Hydroxymethyl-C (-)	1.06
Trihydroxybutyl-C (+)	1.06
Methyl-T (+)	1.09
Trihydroxybutyl-T (+)	1.11
Nitro-C (-)	1.13
Hydroxymethylhydantion (-)	1.15
Nitro-A (-)	1.17
Hydroxyethyl-T* or Methoxymethyl-T* (+)	1.25
Hydroxymethyl-T (+)	1.25
Trihydroxybutyl-T* (-)	1.27
Dihydro-U (+)	1.31
O6-Carboxymethyl-G (+)	1.39
1,N2-propano-G (+)	1.39
Carboxyethyl-A (+)	1.39
Methyl-G or Hydroxymethyl-A (+)	1.44
Dihydro-T (+)	1.49
Nitro-T (-)	1.49
Hydroxyhydro-C (-)	1.53
Tetramethyl-C (+)	1.53
Carboxyethyl-T (-)	1.57
Methyl-G* or Hydroxymethyl-A* (+)	1.67
Trihydroxybutyl-C (-)	1.68
Trihydroxybutyl-C* (+)	1.68
Carboxyhydroxyethyl-A or Carboxyethyl-G or Methylglyoxal-G (+)	1.72
Dimethyl-T or Ethyl-T (+)	1.83
Hydroxymethyl-G (+)	1.83
Hydroxyhydro-C* (-)	1.84
Carboxyethyl-A* (+)	1.87
Nitro-T* (-)	1.87
Carboxyethyl-A* (+)	1.91
Hydroxymethyl-T* (+)	1.91
Methyl-A (+)	1.94
Pentenal-G (+)	2.13
Crotonaldehyde-G (+)	2.15
Carboxymethyl-A (+)	2.27
Methyl-C (+)	2.27
Dimethyl-G or Ethyl-G or Hydroxyethyl-A or Methoxymethyl-A (+)	2.32
Trimethyl-G or (Iso)propyl-G (+)	2.34

Figure 1. Heat map displaying the mean rise (light to dark red) or decrease (light to dark blue) of putative DNA adduct levels (different DNA adduct isomers marked with *(*)(*)) during 48 hours of colonic beef ('B') or chicken ('C') digestion utilizing the fecal inocula of 10 different test subjects (P1 – P10). To facilitate comparison, DNA adduct levels in T0 samples were subtracted from DNA adduct levels in T48 samples, rendering positive values (T48 > T0) to be red, and negative values (T0 > T48) to be blue. Darker shades document larger differences in DNA adduct signal intensity. White boxes represent a difference close to 0, whilst grey boxes represent non-detection of that specific DNA adduct in those specific samples. RT is expressed in min. G, C, T, A and U respectively represent guanine, cytosine, thymine, adenine and uracil. After each DNA adduct name, the ionization mode is indicated; (+) or (-).



Compound	RT
Carboxymethyl-T or Carboxyethyl-U (-)	2.41
Methyl-G** or Hydroxymethyl-A** (+)	2.41
Dihydroxy-A or Hydroxy-G (+)	2.53
Malondialdehyde-x1-C (+)	2.65
N2,3-etheno-G (+)	2.66
Malondialdehyde-x1-G (+)	2.69
Carboxyhydroxyethyl-A* or Carboxyethyl-G* or Methylglyoxal-G* (+)	2.72
Dimethyl-G* or Ethyl-G* or Hydroxyethyl-A* or Methoxymethyl-A* (+)	2.81
Hydroxyethyl-T* or Methoxymethyl-T* (+)	2.83
O6-Methyl-G (+)	2.95
Dimethyl-G** or Ethyl-G** or Hydroxyethyl-A** or Methoxymethyl-A** (+)	2.99
Dihydroxy-A* or Hydroxy-G* (+)	3.12
Hydroxyethyl-G or Methoxymethyl-G (-)	3.27
Formamidopyrimidine-A (-)	3.29
Oxohexanal-C (-)	3.34
Hydroxynonenal-G (+)	3.41
Trimethyl-G* or (Iso)propyl-G* (+)	3.46
Hydroxyethyl-G or Methoxymethyl-G (+)	3.56
Hydroxybutyl-G (+)	3.74
Hydroxynonenal-G* (+)	3.76
Oxohexanal-C* (-)	3.78
3,N4-ethenoC (-)	3.83
Dimethyl-A or Ethyl-A (+)	3.87
Hydroxyethyl-C* or Methoxymethyl-C*(+)	3.99
Trihydroxybutyl-C** (+)	3.99
Hydroxyhydro-G (-)	4.01
Hydroxynonenal-G** (+)	4.03
Trihydroxybutyl-U (+)	4.07
Malondialdehyde-x3-C (+)	4.26
Hydroxynonenal-C (+)	4.32
Hydroxynonenal-C (-)	4.44
Hydroxydihydro-T (+)	4.48
Carbamoylhydroxyethyl-A or Carbamoylethyl-G (+)	4.49
Trihydroxybutyl-T* (+)	4.59
Malondialdehyde-x3-C (-)	4.61
Malondialdehyde-x3-A (-)	4.76
Oxohexanal-methyl-C (-)	4.81
Heptenal-G (-)	4.84
Hydroxyhydro-G* (-)	4.94
Glyoxal-G(+)	5.05
Hydroxybutyl-C (-)	5.43
Carbamoylhydroxyethyl-G* (+)	5.49
Heptenal-etheno-G (-)	5.52
Hydroxy-C (+)	5.53
Malondialdehyde-Acetaldehyde-A (+)	5.56

Figure 1 continued.



Compound	RT
Carbamoylhydroxyethyl-G (+)	0.81
Hydroxyethyl-C or Methoxymethyl-C (+)	0.89
Dimethyl-C or Ethyl-C (+)	0.98
Hydroxyethyl-T or Methoxymethyl-T (+)	1.01
Trihydroxybutyl-U (-)	1.02
Trihydroxybutyl-T (-)	1.04
Carboxyethyl-T (+)	1.06
Hydroxymethyl-C (-)	1.06
Trihydroxybutyl-C (+)	1.06
Methyl-T (+)	1.09
Trihydroxybutyl-T (+)	1.11
Nitro-C (-)	1.13
Hydroxymethylhydantion (-)	1.15
Nitro-A (-)	1.17
Hydroxyethyl-T* or Methoxymethyl-T* (+)	1.25
Hydroxymethyl-T (+)	1.25
Trihydroxybutyl-T* (-)	1.27
Dihydro-U (+)	1.31
O6-Carboxymethyl-G (+)	1.39
1,N2-propano-G (+)	1.39
Carboxyethyl-A (+)	1.39
Methyl-G or Hydroxymethyl-A (+)	1.44
Dihydro-T (+)	1.49
Nitro-T (-)	1.49
Hydroxyhydro-C (-)	1.53
Tetramethyl-C (+)	1.53
Carboxyethyl-T (-)	1.57
Methyl-G* or Hydroxymethyl-A* (+)	1.67
Trihydroxybutyl-C (-)	1.68
Trihydroxybutyl-C* (+)	1.68
Carboxyhydroxyethyl-A or Carboxyethyl-G or Methylglyoxal-G (+)	1.72
Dimethyl-T or Ethyl-T (+)	1.83
Hydroxymethyl-G (+)	1.83
Hydroxyhydro-C* (-)	1.84
Carboxyethyl-A* (+)	1.87
Nitro-T* (-)	1.87
Carboxyethyl-A* (+)	1.91
Hydroxymethyl-T* (+)	1.91
Methyl-A (+)	1.94
Pentenal-G (+)	2.13
Crotonaldehyde-G (+)	2.15
Carboxymethyl-A (+)	2.27
Methyl-C (+)	2.27
Dimethyl-G or Ethyl-G or Hydroxyethyl-A or Methoxymethyl-A (+)	2.32
Trimethyl-G or (Iso)propyl-G (+)	2.34

Figure 2. Heat map displaying the significant ($p < 0.10$) rise (red) or decrease (blue) of mean putative DNA adduct levels (different isomers marked with *(*)(*)) during 48 hours of colonic beef ('B') or chicken ('C') digestion utilizing the fecal inocula of 10 different test subjects (P1 – P10). To facilitate comparison, DNA adduct levels in T0 samples were subtracted from DNA adduct levels in T48 samples, rendering positive values to be red, and negative values to be blue. Grey boxes represent non-significant differences. RT is expressed in min. G, C, T, A and U represent guanine, cytosine, thymine, adenine and uracil respectively. After the DNA adduct names, the charge of each detected compound is indicated; (+) means that that specific DNA adduct type was detected in positive ionization mode, whilst (-) means that specific DNA adduct type was detected in negative ionization mode.

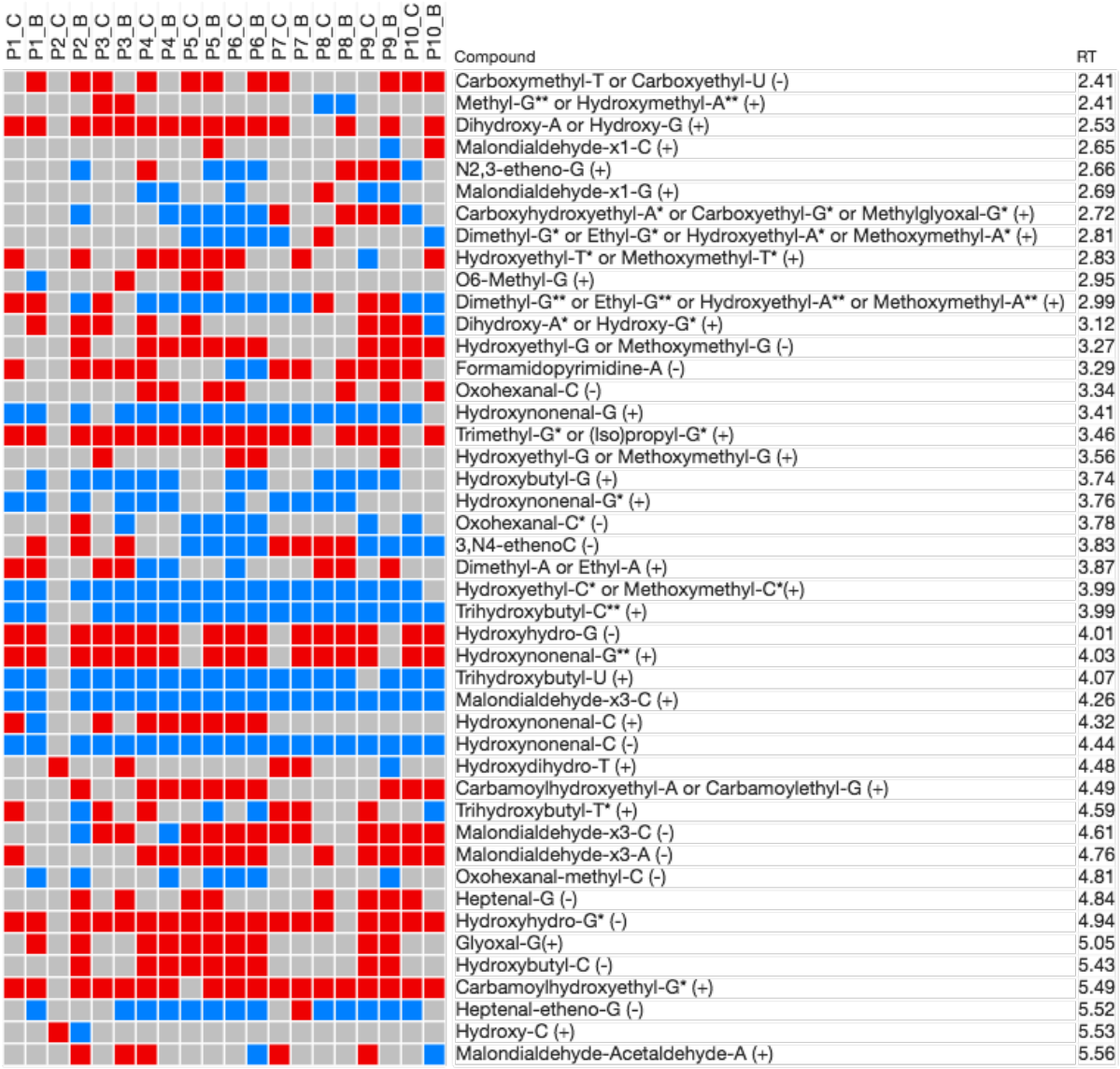
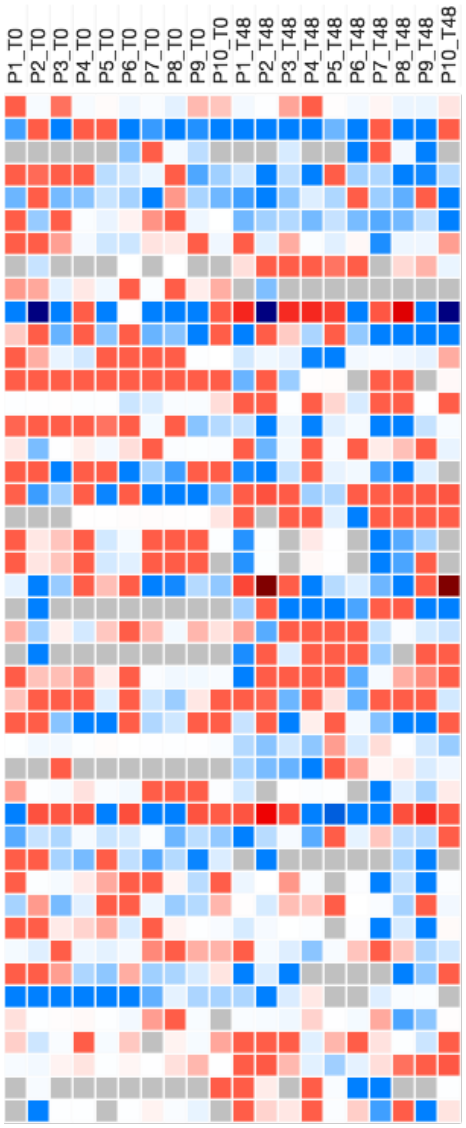
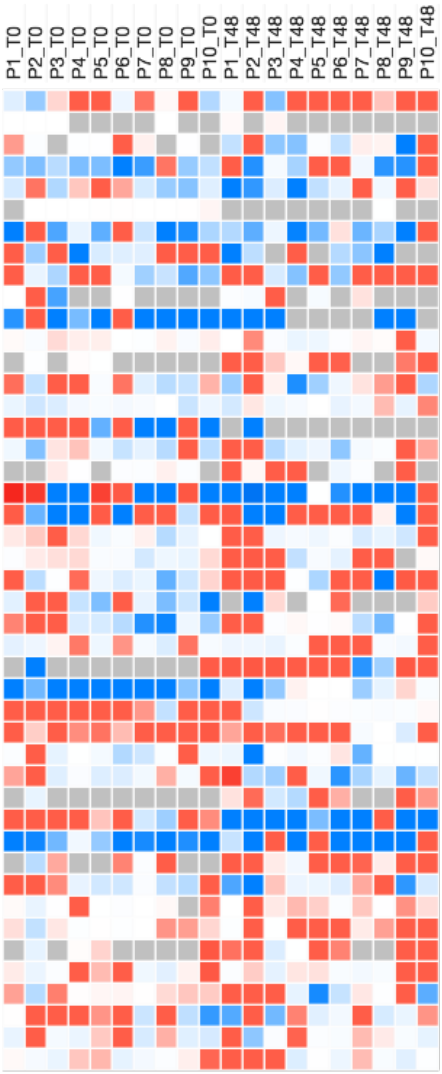


Figure 2 continued.



Compound	RT
Carbamoylhydroxyethyl-G (+)	0.81
Hydroxyethyl-C or Methoxymethyl-C (+)	0.89
Dimethyl-C or Ethyl-C (+)	0.98
Hydroxyethyl-T or Methoxymethyl-T (+)	1.01
Trihydroxybutyl-U (-)	1.02
Trihydroxybutyl-T (-)	1.04
Carboxyethyl-T (+)	1.06
Hydroxymethyl-C (-)	1.06
Trihydroxybutyl-C (+)	1.06
Methyl-T (+)	1.09
Trihydroxybutyl-T (+)	1.11
Nitro-C (-)	1.13
Hydroxymethylhydantion (-)	1.15
Nitro-A (-)	1.17
Hydroxyethyl-T* or Methoxymethyl-T* (+)	1.25
Hydroxymethyl-T (+)	1.25
Trihydroxybutyl-T* (-)	1.27
Dihydro-U (+)	1.31
O6-Carboxymethyl-G (+)	1.39
1,N2-propano-G (+)	1.39
Carboxyethyl-A (+)	1.39
Methyl-G or Hydroxymethyl-A (+)	1.44
Dihydro-T (+)	1.49
Nitro-T (-)	1.49
Hydroxyhydro-C (-)	1.53
Tetramethyl-C (+)	1.53
Carboxyethyl-T (-)	1.57
Methyl-G* or Hydroxymethyl-A* (+)	1.67
Trihydroxybutyl-C (-)	1.68
Trihydroxybutyl-C* (+)	1.68
Carboxyhydroxyethyl-A or Carboxyethyl-G or Methylglyoxal-G (+)	1.72
Dimethyl-T or Ethyl-T (+)	1.83
Hydroxymethyl-G (+)	1.83
Hydroxyhydro-C* (-)	1.84
Carboxyethyl-A* (+)	1.87
Nitro-T* (-)	1.87
Carboxyethyl-A* (+)	1.91
Hydroxymethyl-T* (+)	1.91
Methyl-A (+)	1.94
Pentenal-G (+)	2.13
Crotonaldehyde-G (+)	2.15
Carboxymethyl-A (+)	2.27
Methyl-C (+)	2.27
Dimethyl-G or Ethyl-G or Hydroxyethyl-A or Methoxymethyl-A (+)	2.32
Trimethyl-G or (Iso)propyl-G (+)	2.34

Figure 3. Heat map displaying the difference in putatively detected DNA adduct levels (isomers marked with *(*)(*)) for chicken *vs.* beef digestion in T0 and T48 digestive samples of 10 different test subjects (P1 – P10). DNA adduct levels in chicken digests were subtracted from those in beef digests to enable straightforward comparison. Red indicates higher putative DNA adduct levels in beef digests compared to chicken digests (i.e. concentration in beef > chicken). Blue represents lower putative DNA adduct levels in comparison (subtraction has a negative outcome; i.e. chicken > beef). White boxes represent a difference close to 0, whilst grey boxes represent the fact that that specific DNA adduct was not detected in those specific samples. RT is expressed in min. G, C, T, A and U stand for guanine, cytosine, thymine, adenine and uracil respectively. After each DNA adduct name, the ionization mode is indicated; (+) or (-).



Compound	RT
Carboxymethyl-T or Carboxyethyl-U (-)	2.41
Methyl-G** or Hydroxymethyl-A** (+)	2.41
Dihydroxy-A or Hydroxy-G (+)	2.53
Malondialdehyde-x1-C (+)	2.65
N2,3-etheno-G (+)	2.66
Malondialdehyde-x1-G (+)	2.69
Carboxyhydroxyethyl-A* or Carboxyethyl-G* or Methylglyoxal-G* (+)	2.72
Dimethyl-G* or Ethyl-G* or Hydroxyethyl-A* or Methoxymethyl-A* (+)	2.81
Hydroxyethyl-T* or Methoxymethyl-T* (+)	2.83
O6-Methyl-G (+)	2.95
Dimethyl-G** or Ethyl-G** or Hydroxyethyl-A** or Methoxymethyl-A** (+)	2.99
Dihydroxy-A* or Hydroxy-G* (+)	3.12
Hydroxyethyl-G or Methoxymethyl-G (-)	3.27
Formamidopyrimidine-A (-)	3.29
Oxohexanal-C (-)	3.34
Hydroxynonenal-G (+)	3.41
Trimethyl-G* or (Iso)propyl-G* (+)	3.46
Hydroxyethyl-G or Methoxymethyl-G (+)	3.56
Hydroxybutyl-G (+)	3.74
Hydroxynonenal-G* (+)	3.76
Oxohexanal-C* (-)	3.78
3,N4-ethenoC (-)	3.83
Dimethyl-A or Ethyl-A (+)	3.87
Hydroxyethyl-C* or Methoxymethyl-C*(+)	3.99
Trihydroxybutyl-C** (+)	3.99
Hydroxyhydro-G (-)	4.01
Hydroxynonenal-G** (+)	4.03
Trihydroxybutyl-U (+)	4.07
Malondialdehyde-x3-C (+)	4.26
Hydroxynonenal-C (+)	4.32
Hydroxynonenal-C (-)	4.44
Hydroxydihydro-T (+)	4.48
Carbamoylhydroxyethyl-A or Carbamoylethyl-G (+)	4.49
Trihydroxybutyl-T* (+)	4.59
Malondialdehyde-x3-C (-)	4.61
Malondialdehyde-x3-A (-)	4.76
Oxohexanal-methyl-C (-)	4.81
Heptenal-G (-)	4.84
Hydroxyhydro-G* (-)	4.94
Glyoxal-G(+)	5.05
Hydroxybutyl-C (-)	5.43
Carbamoylhydroxyethyl-G* (+)	5.49
Heptenal-etheno-G (-)	5.52
Hydroxy-C (+)	5.53
Malondialdehyde-Acetaldehyde-A (+)	5.56

Figure 3 continued.

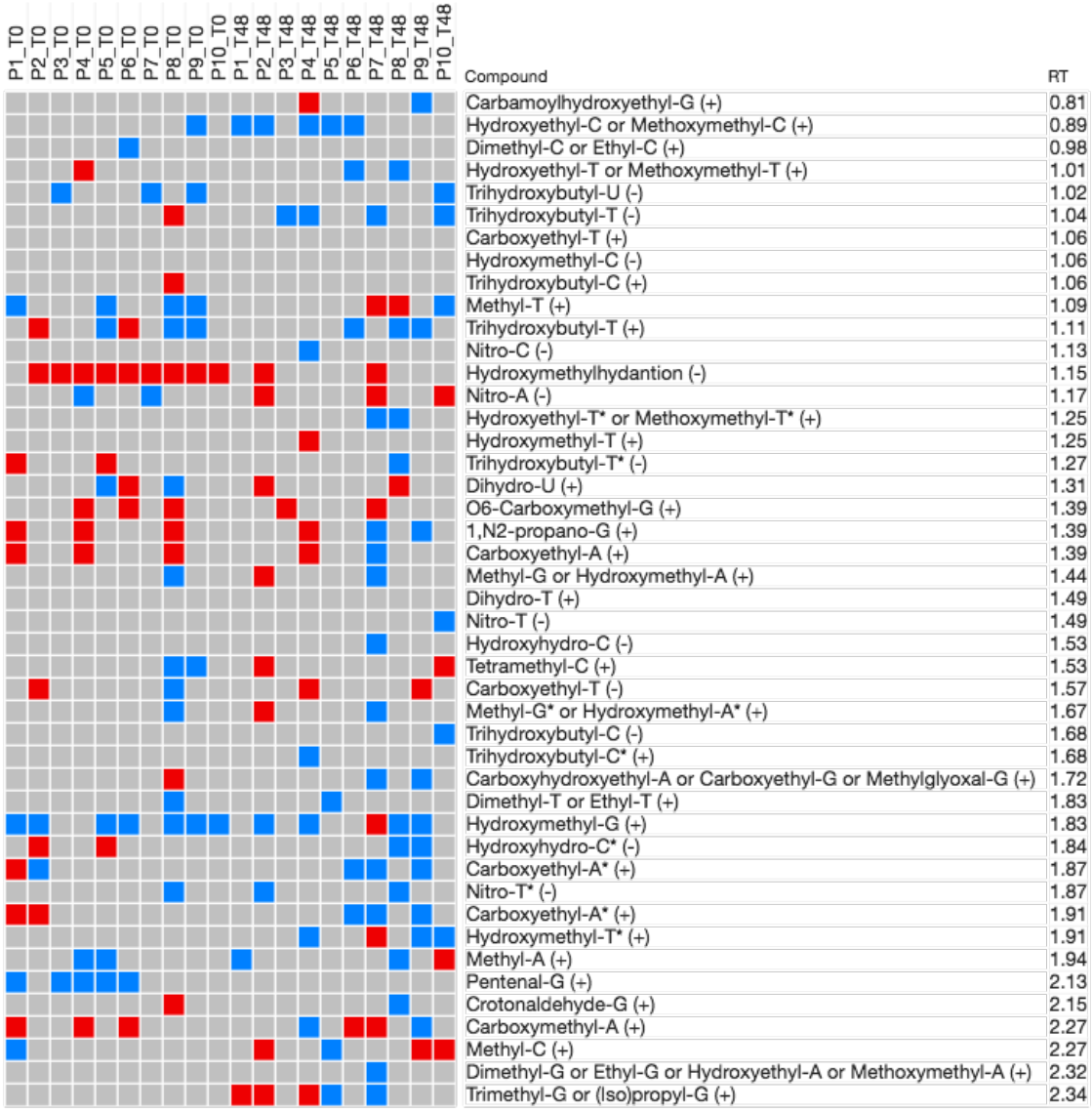


Figure 4. Heat map displaying significant ($p < 0.10$) differences in putatively detected DNA adduct levels (isomers marked with *(*)(*)) for chicken *vs.* beef digestion in T0 and T48 digestive samples by of 10 different test subjects (P1 – P10). DNA adduct levels in chicken digests were subtracted from those in beef digests to enable straightforward comparison. Red indicates significantly higher DNA adduct levels in beef digests compared to chicken digests (subtraction has a positive outcome; beef > chicken). Blue indicates significantly lower putative DNA adduct levels in comparison (subtraction has a negative outcome; chicken > beef). Grey boxes represent non-significant differences. RT is expressed in min. G, C, T, A and U stand for guanine, cytosine, thymine, adenine and uracil respectively. After each DNA adduct name, the charge of the detected compound is indicated; (+) for positive, (-) for negative.

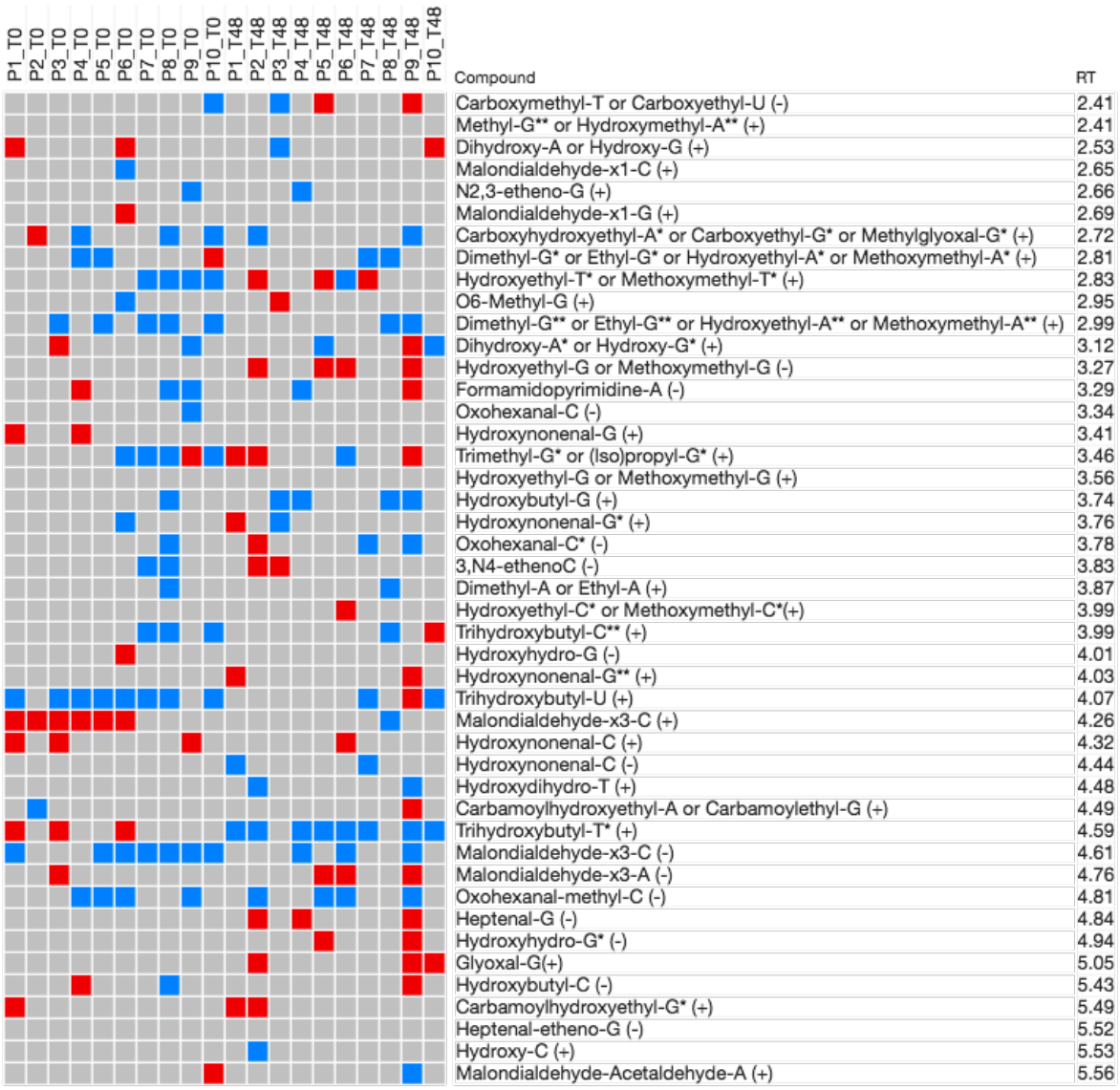


Figure 4 continued.

3.1.2.2 After colonic fermentation (T48)

Differences in DNA adduct formation in T48 beef digests compared to T48 chicken digests are on display in figure 2 as well. DNA adduct levels (peak areas corrected for the amount of guanine in each sample) in chicken digests were subtracted from DNA adduct levels in beef digests. Again, when DNA adduct levels in beef digests were higher compared to chicken digests, this is displayed red, but when DNA adduct levels in chicken digests were higher compared to those in beef digests, this is displayed in blue.

3.2 Multivariate statistics to compare DNA adduct profile in red *vs.* white meat digests

Simca™ analysis was performed to see whether multivariate statistics could be used to discriminate between T0 and T48 samples on the one hand, and beef and chicken digests on the other, as such also enabling the selection of discriminating DNA adduct types.

PCA-X modelling of negative as well as positive ion features revealed a distinct grouping of T0 *vs.* T48 samples (figures 5 and 6), followed by a clear grouping according to test subject. A valid OPLS-DA model discriminating between T0 and T48 samples could be constructed for each test subject separately and all test subjects combined.

Figure 5. Clustering of T0 (orange) *vs.* T48 (blue) digestion samples in the PCA-X plot according to negative ion features.

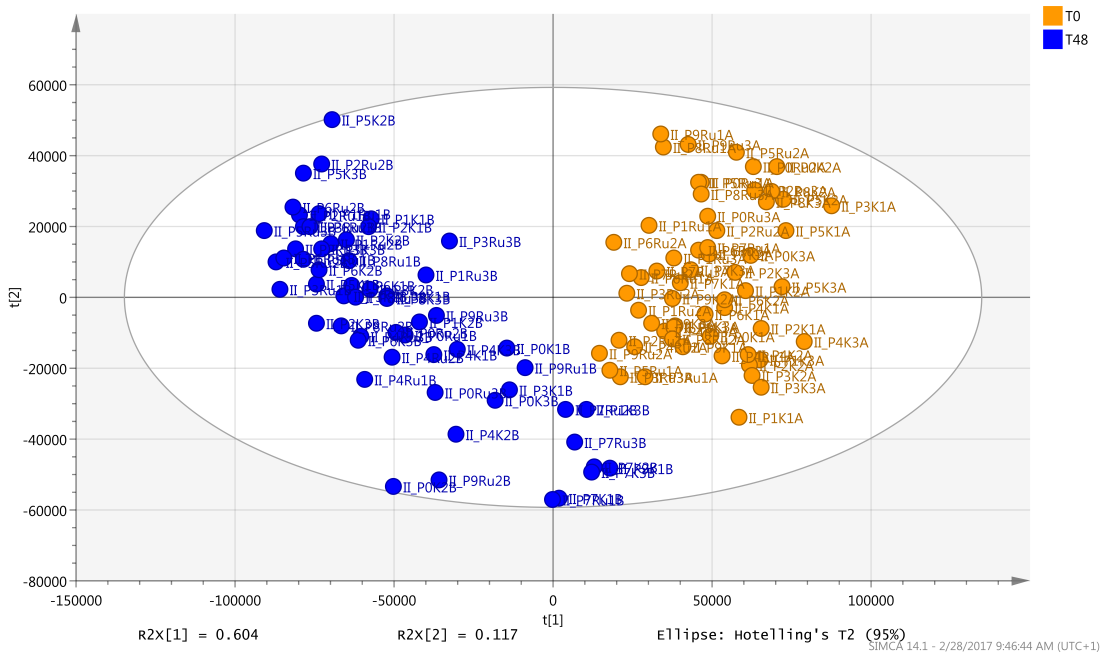


Figure 6. Clustering of T0 (orange) *vs.* T48 (blue) digestion samples in the PCA-X plot according to positive ion features.

During OPLS-DA analysis of positive ion features, a valid model could be constructed to discriminate beef digests from chicken digests overall (for T0 and T48 samples of all 10 test subject digestions), and in T0 samples. This was however not the case for T48 samples. In addition, beef digests could not be discriminated from chicken digests for each test subject separately (data from T0 and T48 samples combined as well as separately), based on information contained in positive as well as negative ion features. With regard to negative ion feature OPLS-DA modelling (using the raw data from all 10 test subject digestions), discrimination between beef *vs.* chicken digests was somewhat clear in T0 samples, but did not suffice to construct a valid OPLS-DA model that met all previously set criteria. OPLS-DA modelling could not be performed for T48 samples either. An overview of the obtained OPLS-DA model characteristics is provided in table 1.

Table 1. OPLS-DA model characteristics.

Model	Charge	Number of components	R^2X	R^2Y	Q^2	CV-ANOVA	Permutation test
T0 *vs.* T48 (beef + chicken samples)	+	1+3+0	0.724	0.983	0.963	$p = 0$	Excellent
T0 *vs.* T48 (beef + chicken samples)	-	1+3+0	0.760	0.971	0.957	$p = 0$	Excellent
Beef *vs.* chicken (T0 + T48 samples)	+	1+9+0	0.830	0.959	0.750	$p < 0.01$	Good
Beef *vs.* chicken (T0)	+	1+6+0	0.715	0.986	0.945	$p < 0.01$	Good
Beef *vs.* chicken (T48)	+	1+3+0	0.296	0.984	0.615	$p < 0.01$	Good
Beef *vs.* chicken (T0 + T48 samples)	-	1+2+0	0.244	0.800	0.236	$p < 0.01$	Good
Beef *vs.* chicken (T0)	-	1+5+0	0.245	0.995	0.633	$p < 0.01$	Good
Beef *vs.* chicken (T48)	-	1+2+0	0.203	0.880	0.255	$p = 0.014$	Sufficient

The valid OPLS-DA model constructed to discriminate between beef and chicken digests in T0 samples (based on positive ion feature data) rendered 4 DNA adduct types with marker potential. These compounds of interest are listed in table 2.

Table 2. DNA adducts discriminating between beef *vs.* chicken digests prior to colonic fermentation.

DNA adduct name	RT (min)	Charge	Δ ppm	Discriminative for	VIP score
Dimethyl-T or ethyl-T	0.72	+	2.92	Beef digests	1.95
Hydroxymethyl-T	0.77	+	3.00	Beef digests	0.89
Methyl-G	1.46	+	3.40	Beef digests	1.23
Tetramethyl-T	2.74	+	3.03	Beef digests	1.66

3.3 The effect of myoglobin digestion on DNA adduct profile

In total, 5 different experiments were set up to decipher the interfering role of myoglobin in red meat genotoxicity. ToxFinder™ profiling was performed for each sample; average putative DNA adduct levels in T0 as well as T48 samples are shown in figure 7. Figure 8 enables correct statistical interpretation of the observed differences.

Next page:

Figure 7. Heat map displaying average putatively detected DNA adduct levels (different isomers are marked with *(*)(*)) in T0 and T48 digestive samples of different digestion experiments. Darker shades of red indicate higher signal intensities. Grey boxes represent the absence of DNA adduct detection in those specific samples. RT is expressed in min. G, C, T, A and U respectively represent guanine, cytosine, thymine, adenine and uracil. After each DNA adduct name, the observed ionization mode is indicated; (+) or (-). "Myo" is short for myoglobin.

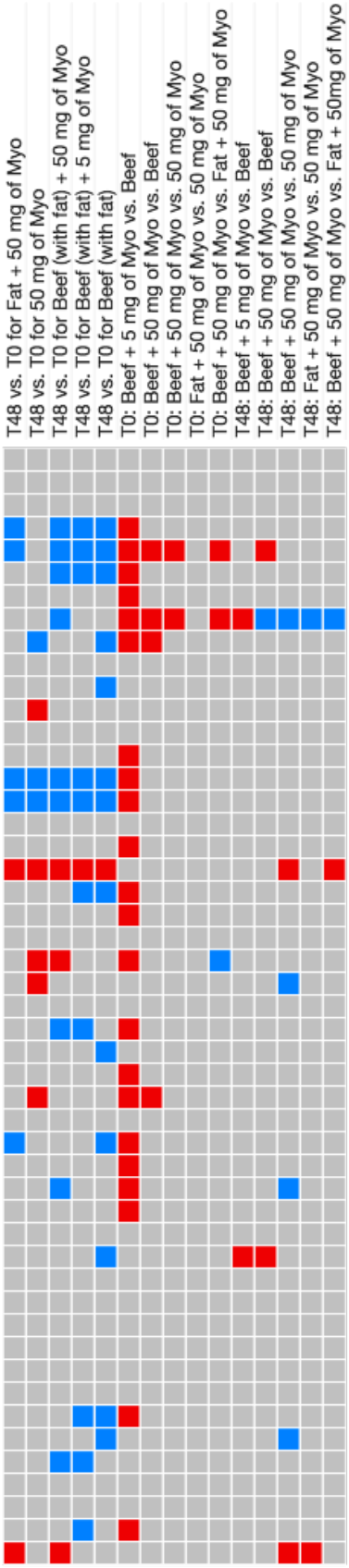
Compound	RT
Hydroxyethyl-C or Methoxymethyl-C (+)	0.87
Carbamoylhydroxyethyl-G (+)	0.91
Hydroxyethyl-C* or Methoxymethyl-C* (+)	0.95
Trihydroxybutyl-U (-)	1.02
Carboxyethyl-T (+)	1.04
Trihydroxybutyl-T (-)	1.05
Dihydro-U (+)	1.09
Hydroxyethyl-T or Methoxymethyl-T (+)	1.09
Trihydroxybutyl-C (+)	1.09
Nitro-C (-)	1.11
Hydroxymethylhydantion (-)	1.13
Methyl-A (+)	1.15
Hydroxymethyl-C (+)	1.17
Methyl-T (+)	1.23
Trihydroxybutyl-T (+)	1.26
Trihydroxybutyl-T* (-)	1.27
Hydroxymethyl-T (+)	1.28
Dihydro-U* (+)	1.31
O6-Carboxymethyl-G (+)	1.48
Carboxyethyl-A (+)	1.59
Methyl-G or Hydroxymethyl-A (+)	1.59
Carboxyethyl-T (-)	1.63
Dihydro-T (+)	1.64
Trihydroxybutyl-C (-)	1.66
Trihydroxybutyl-C* (+)	1.68
1,N2-propano-G (+)	1.75
Carboxyethyl-A* (+)	1.75
Methoxymethyl-G (+)	1.82
Nitro-A (-)	1.82
Hydroxymethyl-G (+)	1.84
Methyl-G* or Hydroxymethyl-A* (+)	1.84
Tetramethyl-C (+)	1.84
Dimethyl-T (+)	1.87
Methoxymethyl-T (+)	1.95
Nitro-T (-)	1.96
Hydroxymethyl-T* (+)	1.98
Methyl-A* (+)	2.23
Pentenal-G (+)	2.26
Carboxymethyl-A (-)	2.34
Methyl-C (+)	2.41
Dihydroxy-A or Hydroxy-G (-)	2.57
Trimethyl-G or (Iso)propyl-G (+)	2.61
Carboxyethyl-G (+)	2.71
Carboxyhydroxyethyl-A or Carboxyethyl-G or Methylglyoxal-G (+)	2.71
Malondialdehyde-x1-G (+)	2.74
N2,3-etheno-G (+)	2.74
Hydroxyethyl-T* or Methoxymethyl-T* (+)	2.84
Malondialdehyde-x1-C (+)	2.86
O6-Methyl-G (+)	2.97

Compound	RT
Dimethyl-G or Ethyl-G or Hydroxyethyl-A or Methoxymethyl-A (+)	3.17
Dihydroxy-A or Hydroxy-G (+)	3.28
Formamidopyrimidine-A (-)	3.29
Oxohexanal-C (-)	3.35
Methoxymethyl-G (-)	3.59
Methoxymethyl-G* (+)	3.59
Trimethyl-G* or (Iso)propyl-G* (+)	3.67
Oxohexanal-C* (-)	3.79
3,N4-etheno-C (-)	3.82
Hydroxybutyl-G (+)	3.86
Hydroxyethyl-C* or Methoxymethyl-C* (+)	3.94
Hydroxyhydro-G (-)	3.99
Dimethyl-A or Ethyl-A (+)	4.08
Hydroxynonenal-G (+)	4.08
Dimethyl-C (+)	4.19
Hydroxynonenal-G* (+)	4.19
Hydroxybutyl-G (-)	4.26
Hydroxynonenal-C (+)	4.31
Hydroxynonenal-C (-)	4.32
Trihydroxybutyl-U (+)	4.42
Hydroxydihydro-T (+)	4.51
Trihydroxybutyl-C** (+)	4.51
Malondialdehyde-x3-C (-)	4.57
Trihydroxybutyl-T* (+)	4.77
Heptenal-G (-)	4.87
Hydroxyhydro-G* (-)	4.91
Oxohexanal-methyl-C (-)	4.95
Glyoxal-G (+)	5.01
Hydroxybutyl-C (-)	5.45
Carbamoylhydroxyethyl-G* (+)	5.48
Hydroxy-C (+)	5.55
Malondialdehyde-Acetaldehyde-A (+)	5.58

Figure 7 continued.

Next page:

Figure 8. Heat map displaying significant ($p < 0.10$) differences in putatively detected DNA adduct levels (different isomers marked with *(*)(*)) in samples from the different experimental setups. Red indicates significantly higher DNA adduct levels in the first compared to the second group mentioned in the observed column. Blue indicates significantly lower putative DNA adduct levels in the first compared to the second group mentioned in the observed column. Grey boxes represent non-significant differences. RT is expressed in min. G, C, T, A and U stand for guanine, cytosine, thymine, adenine and uracil, respectively. After each DNA adduct name, the observed ionization mode is indicated; (+) or (-). "Myo" is short for myoglobin.



Compound	RT
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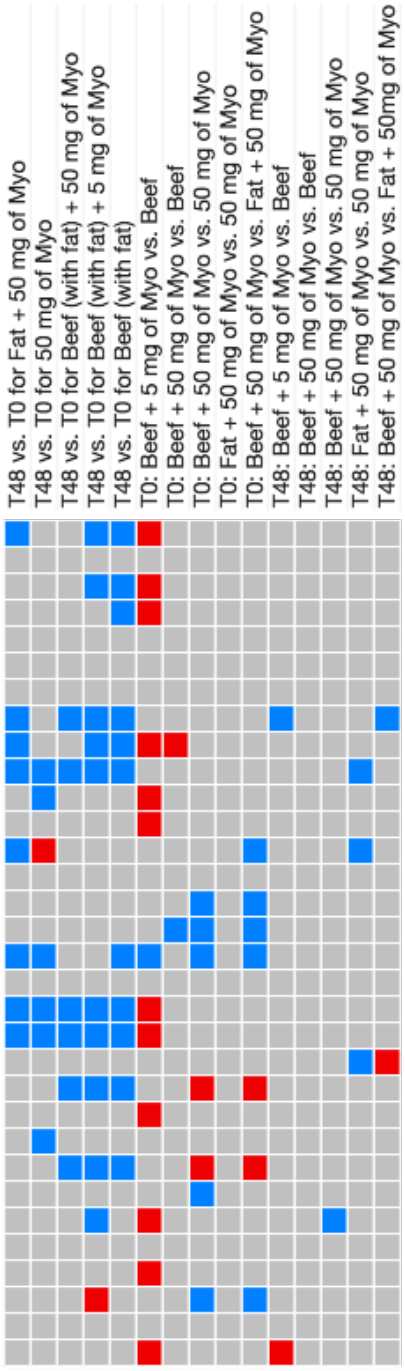
Trihydroxybutyl-U (-)	1.02
Carboxyethyl-T (+)	1.04
Trihydroxybutyl-T (-)	1.05
Dihydro-U (+)	1.09
Hydroxyethyl-T or Methoxymethyl-T (+)	1.09
Trihydroxybutyl-C (+)	1.09
Nitro-C (-)	1.11
Hydroxymethylhydantion (-)	1.13
Methyl-A (+)	1.15
Hydroxymethyl-C (+)	1.17
Methyl-T (+)	1.23
Trihydroxybutyl-T (+)	1.26
Trihydroxybutyl-T* (-)	1.27
Hydroxymethyl-T (+)	1.28
Dihydro-U* (+)	1.31
O6-Carboxymethyl-G (+)	1.48
Carboxyethyl-A (+)	1.59
Methyl-G or Hydroxymethyl-A (+)	1.59
Carboxyethyl-T (-)	1.63
Dihydro-T (+)	1.64
Trihydroxybutyl-C (-)	1.66
Trihydroxybutyl-C* (+)	1.68
1,N2-propano-G (+)	1.75
Carboxyethyl-A* (+)	1.75
Methoxymethyl-G (+)	1.82
Nitro-A (-)	1.82
Hydroxymethyl-G (+)	1.84
Methyl-G* or Hydroxymethyl-A* (+)	1.84
Tetramethyl-C (+)	1.84
Dimethyl-T (+)	1.87
Methoxymethyl-T (+)	1.95
Nitro-T (-)	1.96
Hydroxymethyl-T* (+)	1.98
Methyl-A* (+)	2.23
Pentenal-G (+)	2.26
Carboxymethyl-A (-)	2.34
Methyl-C (+)	2.41
Dihydroxy-A or Hydroxy-G (-)	2.57
Trimethyl-G or (Iso)propyl-G (+)	2.61
Carboxyethyl-G (+)	2.71
Carboxyhydroxyethyl-A or Carboxyethyl-G or Methylglyoxal-G (+)	2.71
Malondialdehyde-x1-G (+)	2.74
N2,3-etheno-G (+)	2.74
Hydroxyethyl-T* or Methoxymethyl-T* (+)	2.84
Malondialdehyde-x1-C (+)	2.86
O6-Methyl-G (+)	2.97



Compound	RT
Dimethyl-G or Ethyl-G or Hydroxyethyl-A or Methoxymethyl-A (+)	3.17
Dihydroxy-A or Hydroxy-G (+)	3.28
Formamidopyrimidine-A (-)	3.29
Oxohexanal-C (-)	3.35
Methoxymethyl-G (-)	3.59
Methoxymethyl-G* (+)	3.59
Trimethyl-G* or (Iso)propyl-G* (+)	3.67
Oxohexanal-C* (-)	3.79
3,N4-etheno-C (-)	3.82
Hydroxybutyl-G (+)	3.86
Hydroxyethyl-C* or Methoxymethyl-C* (+)	3.94
Hydroxyhydro-G (-)	3.99
Dimethyl-A or Ethyl-A (+)	4.08
Hydroxynonenal-G (+)	4.08
Dimethyl-C (+)	4.19
Hydroxynonenal-G* (+)	4.19
Hydroxybutyl-G (-)	4.26
Hydroxynonenal-C (+)	4.31
Hydroxynonenal-C (-)	4.32
Trihydroxybutyl-U (+)	4.42
Hydroxydihydro-T (+)	4.51
Trihydroxybutyl-C** (+)	4.51
Malondialdehyde-x3-C (-)	4.57
Trihydroxybutyl-T* (+)	4.77
Heptenal-G (-)	4.87
Hydroxyhydro-G* (-)	4.91
Oxohexanal-methyl-C (-)	4.95
Glyoxal-G (+)	5.01
Hydroxybutyl-C (-)	5.45
Carbamoylhydroxyethyl-G* (+)	5.48
Hydroxy-C (+)	5.55
Malondialdehyde-Acetaldehyde-A (+)	5.58

Figure 8 continued.

4. DISCUSSION

This study aimed to further unravel the genotoxic effects of red meat consumption due to DNA alkylation and/or oxidation in light of the current hypotheses on the link between red meat consumption and CRC development. It has previously been demonstrated that red meat digestion can promote colon carcinogenesis dependent on heme concentration [29]. The exact underlying mechanisms have not been elucidated yet, but it was hypothesized that heme iron readily catalyzes the formation of genotoxic NOCs and LPOs [4]. To measure the hence induced DNA adduct formation, the in-house DNA adductomics methodology [22] was implemented. The application of this state-of-the-art DNA adductomics platform is highly innovative, allowing us to take the field of DNA adduct research to the next level.

DNA adduct profiling demonstrated a clear inter-individual variability with regard to the types and levels of alkylation and/or oxidation induced DNA adducts at the start as well as shortly after *in vitro* colonic meat fermentation. This was reflected by ToxFinder™ profiling as well as Simca™ modelling, and is perfectly in line with previous findings [24, 30]. Prior to the start of each colonic fermentation, a fecal inoculum is added, resulting in the cultivation of a certain individual's colorectal microbiota in order to mimic colonic meat fermentation after enzymatic stomach and small bowel digestion. As such, a pre-colonic digestive sample (T0) can contain DNA adducts because of (a) the interaction between the added fecal DNA (from human, bacterial or dietary origin) and genotoxic molecules formed during the small intestinal digestion of meat, or (b) their presence in the (pre-cultivated) fecal inoculum itself due to prior *in vivo* formation [24]. For example, we have previously demonstrated that the presence of O^6-CMG, an alkylation induced DNA adduct, in meat digests can be linked back to the fecal donor. In other words, some fecal inocula contain and/or lead to the active production of O^6-CMG prior to and/or during colonic fermentation, whilst others simply do not [24, 30]. A rise in DNA adduct levels during colonic fermentation suggests active formation of its precursor molecules by the colonic microbiota (e.g. O^6-CMG does no longer rise if the fecal microbiome is neutralized due to autoclavation [24]), whilst a decrease indicates active or passive degradation. Indeed, it has become indisputable that the gut microbiome exerts beneficial as well as detrimental effects on gut and overall human health. With regard to CRC, it has become clear that the gut microbiome actively contributes to cell proliferation, apoptosis, differentiation, and DNA damage. As a result, the gut microbiome, and its metabolic products, strongly influence whether someone develops

CRC, or not [12, 31]. However, due to the complexity of host-diet-microbiome interactions, a lot of questions still remain [31], especially concerning gut microbiome induced DNA adduct formation.

In this study, several oxidation and/or alkylation induced DNA adducts could be detected prior to colonic fermentation (at 'T0') as well as at the end of digestion (at 'T48'). The number of putatively identified DNA adducts that significantly increased during colonic meat fermentation exceeds 60, and e.g. includes Methyl-T (RT 1.09 min) in test subjects 3, 4, 5, 6 and 9; and Methyl-G (or its Hydroxymethyl-A isomer, RT 1.44 min) in all 10 test subjects. In contrast, over 40 tentatively identified DNA adduct types demonstrate a significant decline during colonic fermentation. The latter DNA adducts types are not of specific interest in T48 samples, but could be relevant due to their natural *in vivo* occurrence and/or *in vitro* formation following small intestinal meat digestion (in T0 samples). Therefore, specific attention should be given to DNA adduct types that are more prevalent in beef digests compared to chicken digests if present in T0 samples overall, and T48 samples if the DNA adduct of interest rises during colonic fermentation.

DNA adduct types demonstrating a significantly higher formation due to the digestion of beef compared to chicken are of specific interest because those specific DNA adduct types could be of importance with regard to CRC initiation through N-nitrosation and lipid peroxidation processes during red meat digestion. However, since not all DNA adduct types are (as) promutagenic and/or procarcinogenic (e.g. 7-MeG is not mutagenic, whilst O^6-MeG is [32]), the reported *in vitro* observations require linkage to (intermediate) effects and/or disease outcome *in vivo*. Unfortunately, the DNA adductomics methodology/technology has not yet been implemented in *in vivo* CRC studies. Hence, at the time being, information on *in vivo* DNA adduct formation in relation to CRC is quite limited. Nevertheless, it has e.g. previously been demonstrated that normal colonic tissue of CRC patients contains significantly higher DNA adduct levels compared to colonic tissue from healthy controls [33].

To the best of our knowledge, O^6-CMG is the only DNA adduct type that has directly been associated with red meat consumption *in vivo*, which we have been able to confirm *in vitro* in this and in previous studies; i.e. O^6-CMG significantly rises upon red meat digestion and myoglobin addition [24]. Since we already know that O^6-CMG is actively formed by the colonic microbiota

during colonic meat fermentation [24], the results of this study are able to confirm and emphasize the relevance of O^6-CMG formation in relation to red meat digestion in the (human) colon.

Apart from O^6-CMG, we were able to detect several other putatively identified DNA adduct types that could be related to red meat digestion *in vitro* as well as *in vivo*. However, in-depth discussion of each individual DNA adduct type is not feasible. Therefore, we will focus on the most prominent significant findings.

Multivariate statistics delivered dimethyl-T (or ethyl-T), hydroxymethyl-T and tetramethyl-T and methylguanine (MeG) as potential DNA alkylation (and also oxidation in case of hydroxymethyl-T) red meat digestion markers. MeG DNA adducts are among the most commonly studied alkylation DNA adduct types. In contrast to our previous *in vitro* meat digestion studies, we were able to detect O^6-MeG in pre- and post-colonic meat digests. More specifically, O^6-MeG significantly increased during colonic meat digestions performed with 4 out of 10 fecal inocula. A clear trend with regard to red *vs.* white meat digestions could however not be observed. We were also able to detect 3 methylated G residues besides O^6-MeG; MeG isomers eluting at 1.44, 1.67 and 2.41 min respectively. The first isomer (RT 1.44 min) is most striking since it could be detected for all 10 test subjects. At first glance, there was no distinct pattern according to digested meat type. Addition of myoglobin to beef digestion seemed to increase the concentration of this specific MeG isomer, although not significantly ($p > 0.10$). Nevertheless, multivariate statistics labeled this MeG isomer as a discriminative molecule for beef digestion across all 10 test subjects. Because this MeG isomer demonstrated the highest signal intensities, it most likely corresponds to 7-MeG, the most prominently formed DNA alkylation lesion that has previously been detected *in vivo*, but is not promutagenic [7, 32]. On the other hand, DNA methylation does regulate gene expression, whilst the presence of 7-MeG in a DNA sequence can also prematurely end DNA replication [7], demonstrating the *in vivo* relevance of 7-MeG as a DNA alkylation marker. Information on the *in vivo* as well as *in vitro* formation of dimethyl-T (or Ethyl-T), hydroxymethyl-T and tetramethyl-T in the context of food digestion and/or cancer development is negligible at the time being, apart from dimethyl-T, which can alternatively correspond to ethyl-T. More specifically, e.g. O^4-ethylthymine (O^4-eT) has previously been detected *in vivo* and linked to the daily exposure to ethylating agents [34, 35]. We know that O^4-

eT can induce DNA miscoding, rendering O^4-eT to be a compound of interest in the context of cancer initiation [7]. Huh et al. furthermore documented that this compound was significantly more present in malignant liver tumors compared to non-tumoral tissue [34]. Hence, the retrieved dimethyl-T or ethyl-T adduct definitely is a compound of interest.

Based on ToxFinder™ screening it could be observed that the hydroxymethylhydantion and malondialdehyde-3x-C DNA adducts were significantly higher ($p < 0.05$) in T0 beef digests compared to chicken digests for at least 6 test subjects. Hydroxymethylhydantion, a ROS induced T alteration [36], appeared to be significantly higher after small bowel digestion of beef in comparison to chicken for all 10 test subjects but P1, suggesting that small bowel beef digestion induced oxidative stress and ROS production. In contrast, myoglobin addition did not significantly increase hydroxymethylhydantion levels. A DNA adduct type with a highly similar behavioral pattern is malondialdehyde-3x-C (M_3C, eluting at 4.26 min); i.e. M_3C was significantly higher in T0 beef digests for 6 test subjects. M_3C is a cytosine analogue formed due to the interaction with 3 MDA molecules, whilst MDA itself is a well-known LPO [37, 38]. In previous work, we were able to demonstrate that (lipid) peroxidation primarily occurs prior to colonic fermentation [24, 39]. Therefore, the retrieval of ROS and/or LPO induced DNA adducts in pre-colonic digestion samples is perfectly in line with expectations. A similar trend for hydroxymethylhydantion, malondialdehyde-3x-C or any other DNA adduct types could not be observed in T48 samples.

In total, 34 different alkylation and/or oxidation induced DNA adduct types significantly ($p < 0.10$) increased in pre-colonic digestion samples upon addition of (5 mg of) myoglobin (observed by means of Toxfinder™ data processing). Of these specific DNA adduct types, hydroxyethyl-T (or methoxymethyl-T), carboxyethyl-T and 3,N^4-etheno-C, demonstrated significant potential as heme-rich meat digestion markers. Hydroxyethyl-T (eluting shortly after 1 min), which might alternatively correspond to methoxymethyl-T, appeared to be higher in digests of beef compared to chicken for 5 different fecal inocula ($p > 0.10$ for 4 out of 5 and $p = 0.0003$ for 1 out of 5), and significantly increased due to addition and digestion of myoglobin; $p = 0.034$ for 5 mg of added myoglobin, and $p = 0.007$ for 50 mg of added myoglobin. The same trend could be observed for carboxyethyl-T (eluting shortly after 1 min) and 3,N^4-etheno-C (RT of 3.83 min). Carboxyethyl-T was higher ($p > 0.10$) in T0 beef digestion samples (compared to chicken) for 6

out of 10 test subjects, and furthermore significantly rose upon myoglobin addition ($p = 0.056$ for 5 mg of myoglobin, $p = 0.004$ for 50 mg of myoglobin). 3,N^4-etheno-C was higher ($p < 0.10$) in beef digests using 4 out of 10 fecal inocula, and also significantly increased due to the digestion of added myoglobin ($p= 0.003$ for 5 mg, and $p = 0.098$ for 50 mg). For certain fecal inocula/test subjects, these particular DNA adduct types were also (significantly) higher ($p < 0.10$) in post-colonic beef digests (compared to chicken), although myoglobin addition did not significantly influence post-colonic DNA adduct levels. Carboxyethyl-T, formed by alkylation of T, has not previously been detected *in vivo*, but has been synthetized *in vitro* [40]. Hydroxyethyl-T, or its methoxymethyl-T analogue, is best known for its potential antiviral properties [41] but has, to the best of our knowledge, never been linked to *in vivo* environmental genotoxicity. In contrast, hydroxyethyl-G has previously been detected *in vivo*, and is furthermore known to originate from several possible sources including lipid peroxidation [7]. Hydroxyethyl-T may very well have a similar origin. 3,N^4-etheno-C is a known lipid peroxidation induced DNA adduct type that has previously been detected *in vivo* and has furthermore been associated with oxidative stress, base pair substitution mutations and an increased cancer risk [7]. Hence, the retrieval of these DNA adduct types is in support of the heme hypothesis, furthermore providing clues with regard to the underlying pathways.

Throughout this study, the genotoxic effects of beef seemed to be more pronounced in pre-colonic digests in comparison to post-colonic digests. We notice that this is most likely due to a larger variety in catabolic as well as anabolic reactions in the (simulated) large bowel. After all, the large bowel is a reaction vessel, subject to a highly diverse range of microbiotic activities [12], whilst digestion in the stomach and small bowel mainly consist of purely mechanical and chemical reactions and interactions, which are furthermore assumed to be identical throughout the entire experimental setup in this study. As a result, overall variation is considerably lower in pre-colonic meat digestion samples compared to post-colonic digestion samples, attributing to the fact that the genotoxicity of beef could not always be confirmed in post-colonic meat digestion samples.

In conclusion, it has been demonstrated that the use of a DNA adductomics platform, furthermore implementing an in-house DNA adduct database, allowed mapping of diet-related DNA adducts in red *vs.* white meat digests. Different NOC- and LPO-related DNA adduct types

could be tentatively identified. More importantly, these specific DNA adduct types could be relevant with regard to red meat and/or heme genotoxicity, and the hence associated CRC risk. Unfortunately, the available information on the *in vivo* occurrence of a large variety of these DNA adduct types and their relevance in the context of cancer risk, is mostly lacking at the time. Therefore, the *in vivo* relevance of the in this study retrieved DNA adduct types and levels awaits further confirmation. Nevertheless, the results of this study have aided with the exploration of red meat and/or heme induced genotoxicity, and can furthermore be used as future reference for *in vivo* DNA adduct profiling studies.

REFERENCES

[1] B. Stewart, C.P. Wild, editors, World Cancer Report 2014 International Agency for Research on Cancer, WHO2014.
[2] M.C. Poirier, Chemical-induced DNA damage and human cancer risk, Nat Rev Cancer, 4 (2004) 630-637.
[3] IARC, IARC Monographs evaluate consumption of red meat and processed meat, Lyon, 2015.
[4] N.M. Bastide, F. Chenni, M. Audebert, R.L. Santarelli, S. Tache, N. Naud, M. Baradat, I. Jouanin, R. Surya, D.A. Hobbs, G.G. Kuhnle, I. Raymond-Letron, F. Gueraud, D.E. Corpet, F.H. Pierre, A central role for heme iron in colon carcinogenesis associated with red meat intake, Cancer Res, 75 (2015) 870-879.
[5] D. Demeyer, B. Mertens, S. De Smet, M. Ulens, Mechanisms Linking Colorectal Cancer to the Consumption of (Processed) Red Meat: A Review, Critical reviews in food science and nutrition, 56 (2016) 2747-2766.
[6] L.Y. Hemeryck, L. Vanhaecke, Diet-related DNA adduct formation in relation to carcinogenesis, Nutr Rev, 74 (2016) 475-489.
[7] R. De Bont, N. van Larebeke, Endogenous DNA damage in humans: a review of quantitative data, Mutagenesis, 19 (2004) 169-185.
[8] O.C. Martin, M. Chevalier, S. Tache, N. Naud, B. Salles, G. Mirey, F.H. Pierre, E. Boutet-Robinet, In vivo genotoxic effects of dietary heme iron on rat colon mucosa and ex vivo effects on colon cells monitored by an optimized alkaline comet assay, Frontiers in Genetics, 11th International Comet Assay Workshop (2015).
[9] W. Lijinsky, N-Nitroso compounds in the diet, Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 443 (1999) 129-138.
[10] W. Lijinsky, Chemistry and biology of N-nitroso compounds, Cambridge Monographs on Cancer Research, (1992) 480.
[11] P.C. Dedon, S.R. Tannenbaum, Reactive nitrogen species in the chemical biology of inflammation, Archives of Biochemistry and Biophysics, 423 (2004) 12-22.

[12] P. Louis, G.L. Hold, H.J. Flint, The gut microbiota, bacterial metabolites and colorectal cancer, Nat Rev Micro, 12 (2014) 661-672.
[13] A.J. Cross, J.R. Pollock, S.A. Bingham, Haem, not protein or inorganic iron, is responsible for endogenous intestinal N-nitrosation arising from red meat, Cancer Res, 63 (2003) 2358-2360.
[14] A.M. Joosen, G.G. Kuhnle, S.M. Aspinall, T.M. Barrow, E. Lecommandeur, A. Azqueta, A.R. Collins, S.A. Bingham, Effect of processed and red meat on endogenous nitrosation and DNA damage, Carcinogenesis, 30 (2009) 1402-1407.
[15] S.A. Bingham, R. Hughes, A.J. Cross, Effect of White Versus Red Meat on Endogenous N-Nitrosation in the Human Colon and Further Evidence of a Dose Response, The Journal of nutrition, 132 (2002) 3522S-3525S.
[16] R. Hughes, A.J. Cross, J.R. Pollock, S. Bingham, Dose-dependent effect of dietary meat on endogenous colonic N-nitrosation, Carcinogenesis, 22 (2001) 199-202.
[17] G.G. Kuhnle, S.A. Bingham, Dietary meat, endogenous nitrosation and colorectal cancer, Biochemical Society transactions, 35 (2007) 1355-1357.
[18] F. Drabløs, E. Feyzi, P.A. Aas, C.B. Vaagbø, B. Kavli, M.S. Bratlie, J. Peña-Diaz, M. Otterlei, G. Slupphaug, H.E. Krokan, Alkylation damage in DNA and RNA—repair mechanisms and medical significance, DNA Repair, 3 (2004) 1389-1407.
[19] J. Kanner, Oxidative processes in meat and meat products: Quality implications, Meat science, 36 (1994) 169-189.
[20] N.M. Bastide, F.H. Pierre, D.E. Corpet, Heme iron from meat and risk of colorectal cancer: a meta-analysis and a review of the mechanisms involved, Cancer prevention research (Philadelphia, Pa.), 4 (2011) 177-184.
[21] L.J. Marnett, Inflammation and Cancer: Chemical Approaches to Mechanisms, Imaging, and Treatment, The Journal of Organic Chemistry, 77 (2012) 5224-5238.
[22] L.Y. Hemeryck, A.I. Decloedt, J. Vanden Bussche, K.P. Geboes, L. Vanhaecke, High resolution mass spectrometry based profiling of diet-related deoxyribonucleic acid adducts, Anal Chim Acta, 892 (2015) 123-131.
[23] K. Molly, M.V. Woestyne, I.D. Smet, W. Verstraete, Validation of the Simulator of the Human Intestinal Microbial Ecosystem (SHIME) Reactor Using Microorganism-associated Activities, Microbial Ecology in Health and Disease, 7 (1994) 191-200.
[24] J. Vanden Bussche, L.Y. Hemeryck, T. Van Hecke, G.G. Kuhnle, F. Pasmans, S.A. Moore, T. Van de Wiele, S. De Smet, L. Vanhaecke, O(6)-carboxymethylguanine DNA adduct formation and lipid peroxidation upon in vitro gastrointestinal digestion of haem-rich meat, Molecular nutrition & food research, 58 (2014) 1883-1896.
[25] T. Van Hecke, J. Vanden Bussche, L. Vanhaecke, E. Vossen, J. Van Camp, S. De Smet, Nitrite curing of chicken, pork, and beef inhibits oxidation but does not affect N-nitroso compound (NOC)-specific DNA adduct formation during in vitro digestion, J Agric Food Chem, 62 (2014) 1980-1988.
[26] J. Vanden Bussche, S.A. Moore, F. Pasmans, G.G. Kuhnle, L. Vanhaecke, An approach based on ultra-high pressure liquid chromatography-tandem mass spectrometry to quantify O6-methyl and O6-carboxymethylguanine DNA adducts in intestinal cell lines, Journal of chromatography. A, 1257 (2012) 25-33.
[27] M.N. Triba, L. Le Moyec, R. Amathieu, C. Goossens, N. Bouchemal, P. Nahon, D.N. Rutledge, P. Savarin, PLS/OPLS models in metabolomics: the impact of permutation of dataset rows on the K-fold cross-validation quality parameters, Molecular bioSystems, 11 (2015) 13-19.

[28] L. Van Meulebroek, A Metabolomic Framework to Study the Regulating Role of Phytohormones Towards Carotenoids in Tomato Fruit, PhD Thesis, Ghent University, Merelbeke, Belgium, 2014.
[29] F. Pierre, A. Freeman, S. Tache, R. Van der Meer, D.E. Corpet, Beef meat and blood sausage promote the formation of azoxymethane-induced mucin-depleted foci and aberrant crypt foci in rat colons, The Journal of nutrition, 134 (2004) 2711-2716.
[30] L.Y. Hemeryck, C. Rombouts, T. Van Hecke, L. Van Meulebroek, J. Vanden Bussche, S. De Smet, L. Vanhaecke, In vitro DNA adduct profiling to mechanistically link red meat consumption to colon cancer promotion, Toxicology Research, 5 (2016) 1346-1358.
[31] T. Irrazábal, A. Belcheva, Stephen E. Girardin, A. Martin, Dana J. Philpott, The Multifaceted Role of the Intestinal Microbiota in Colon Cancer, Molecular Cell, 54 (2014) 309-320.
[32] A.C. Povey, A.F. Badawi, D.P. Cooper, C.N. Hall, K.L. Harrison, P.E. Jackson, N.P. Lees, P.J. O'Connor, G.P. Margison, DNA Alkylation and Repair in the Large Bowel: Animal and Human Studies, The Journal of nutrition, 132 (2002) 3518S-3521S.
[33] A. Pfohl-Leszkowicz, Y. Grosse, V. Carriere, P.H. Cugnenc, A. Berger, F. Carnot, P. Beaune, I. de Waziers, High levels of DNA adducts in human colon are associated with colorectal cancer, Cancer Res, 55 (1995) 5611-5616.
[34] N. Huh, M.S. Satoh, J. Shiga, T. Kuroki, Detection of O4-ethylthymine in human liver DNA, IARC Sci Publ, (1988) 292-295.
[35] H.-i. Kang, C. Konishi, T. Kuroki, N.-h. Huh, Detection of O6-methylguanine, O4-methylthymine and O4-ethylthymine in human liver and peripheral blood leukocyte DNA, Carcinogenesis, 16 (1995) 1277-1280.
[36] M.S. Cooke, M.D. Evans, M. Dizdaroglu, J. Lunec, Oxidative DNA damage: mechanisms, mutation, and disease, The FASEB Journal, 17 (2003) 1195-1214.
[37] K. Stone, A. Uzieblo, L.J. Marnett, Studies of the reaction of malondialdehyde with cytosine nucleosides, Chemical research in toxicology, 3 (1990) 467-472.
[38] L.J. Marnett, Lipid peroxidation-DNA damage by malondialdehyde, Mutation research, 424 (1999) 83-95.
[39] T. Van Hecke, E. Vossen, J. Vanden Bussche, K. Raes, L. Vanhaecke, S. De Smet, Fat content and nitrite-curing influence the formation of oxidation products and NOC-specific DNA adducts during in vitro digestion of meat, PloS one, 9 (2014) e101122.
[40] A. Segal, J.J. Solomon, U. Maté, Isolation of 3-(2-carboxyethyl)thymine following in vitro reaction of β-propiolactone with calf thymus DNA, Chemico-Biological Interactions, 29 (1980) 335-346.
[41] G. Wang, W.E. Seifert, Synthesis and evaluation of oligodeoxynucleotides containing 4'-C-substituted thymidines, Tetrahedron Letters, 37 (1996) 6515-6518.

CHAPTER VI

Shifts in the rat DNA adductome after consumption of high-heme and/or high-fat meat

Adapted from:

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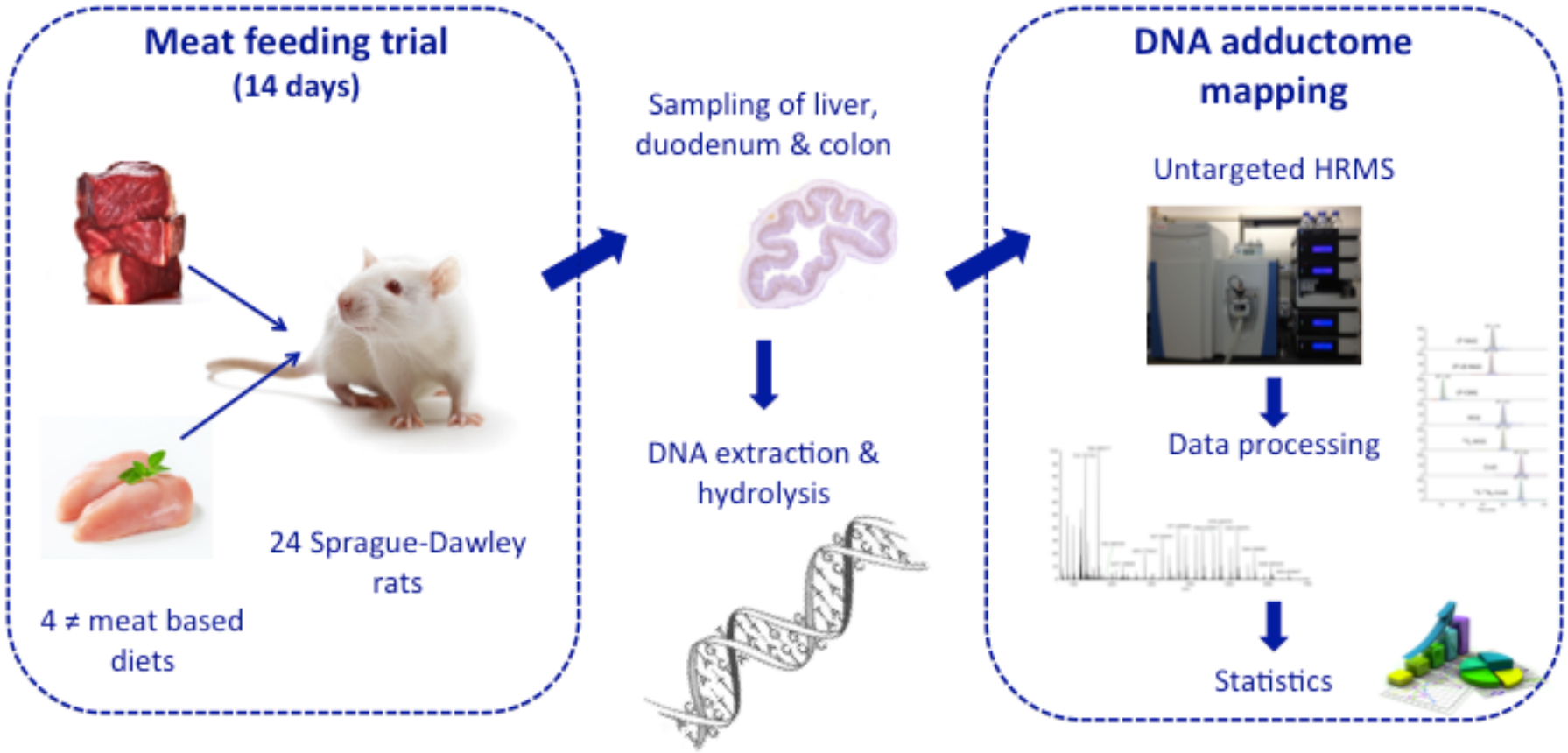
ABSTRACT

Digestion of red and processed meat has been linked to the formation of genotoxic NOCs and LPOs in the gut. In this study, rats were fed a meat based diet to compare the possible genotoxic effects of red *vs.* white meat, and the interfering role of dietary fat. To this purpose, liver, duodenum and colon DNA adductome were analyzed with UHPLC-HRMS. The results demonstrate that the consumed meat type alters the DNA adductome; the levels of 22 different DNA adduct types significantly increased upon the consumption of beef (compared to chicken) and/or lard supplemented beef or chicken. Furthermore, since the retrieved DNA adduct types originate from DNA alkylation, nitrosation and/or oxidation, their formation may be linked to the formation of NOCs and LPOs upon red (and processed) meat digestion, which is in line with the current hypotheses on the causal link between red and processed meat consumption and the development of colorectal cancer.

Keywords:

DNA damage, Dietary fat, High resolution mass spectrometry, Lipid peroxidation products, N-nitroso compounds, Red meat

GRAPHICAL ABSTRACT



1. INTRODUCTION

DNA adduct formation is the result of the attack of nucleophilic sites in DNA by endo- or exogenous electrophilic molecules. As such, the DNA building blocks, i.e. the guanine (G), cytosine (C), adenine (A) and thymine (T) nucleobases can be altered both structurally and functionally. In the absence of a timely detoxification of the initiating genotoxin and/or repair of the resulting DNA adduct, DNA adduct formation can lead to mutations and chemically induced carcinogenesis [1]. Hence, investigation of DNA adduct formation can provide valuable information on exposure to both environmental and endogenous chemicals with genotoxic, mutagenic and/or carcinogenic properties on the one hand, and their possible adverse health effects on the other. For example, DNA adduct formation is believed to be an intermediate step in hepatocarcinogenesis due to chronic aflatoxin B1 exposure. Aflatoxin B1 is a known human carcinogen that is formed as a secondary metabolite by food and feed contaminating fungi. Its uptake results in the formation of different types of DNA adducts and also leads to a correlated increase in liver cancer risk [2]. Accordingly, DNA adduct analysis can be very useful to investigate the underlying pathways of several non-hereditary cancers, which actually comprehends the vast majority of cancer cases [3].

One of the most prevalent cancer types that mainly occurs due to environmental factors (e.g. diet and lifestyle) is colorectal cancer (CRC). CRC is the third and second most common cancer type in men and women worldwide , and important influencing factors include adoption of the Western dietary pattern with the excessive consumption of fat and red and processed meat [3]. With regard to the observed increase in CRC risk due to red and processed meat consumption, different research groups have investigated the proposed underlying pathways. Currently, there are several intertwined hypotheses that are still under investigation. A prominent hypothesis states that heme stimulates the formation of both LPOs and NOCs in the gut besides its own direct (cyto)toxicity [4]. The heme molecule is intrinsically more present in red (e.g. beef) than white (e.g. chicken) meat in the form of myoglobin, which renders this molecule a very potent candidate to help explain the toxicity of red but not white meat. Both exo- and endogenous NOCs may contribute to red and processed meat toxicity. Several types of NOCs (e.g. nitrosamines and nitrosamides) have known carcinogenic properties [5], and the most common route of exposure to NOCs indeed occurs *via* Western type foodstuffs [6]. However, certain NOCs, i.e. nitrosamines and nitrosamides, can also be formed in the gut during digestion of food. What further supports the NOC hypothesis is the fact that exposure to NOCs has already

been linked to an increase in tumor development [5]. The same reasoning actually applies for LPOs; LPOs can originate from both exo- and endogenous processes and possess known cyto- and genotoxic effects that have been linked to carcinogenesis [7, 8].

In previous studies, we were able to link red meat digestion to the increased formation of LPOs (e.g. MDA), as well as LPO- and NOC-related DNA adducts (e.g. O^6-CMG), [9, 10]. The current study aimed to further explore the possible genotoxic effects of red meat consumption *in vivo* since (a) both NOCs and LPOs are prone to DNA adduct formation [8] and (b) a shift in DNA adduct profile after beef digestion has been demonstrated previously *in vitro* [10].

A state-of-the-art DNA adductomics methodology [11], based on accurate mass measurements (HRMS), was employed to map the diet-related DNA adduct profile in tissue from rats on a meat diet. The use of an in-house DNA adduct database and specialized omics software further enabled a focused investigation of possibly relevant diet-related DNA adducts [11].

2. MATERIAL AND METHODS

2.1 Rat feeding trial

2.1.1 Meat based diets

Four different diets, based on lean chicken (LFCh), fat chicken (lean chicken with added lard; HFCh), lean beef (LFBe) or fat beef (lean beef with added lard; HFBe), were prepared in advance. To this purpose, the *m. pectoralis profundus* of chicken, as a model for white meat, and the *m. pectoralis profundus* of beef, as a model for red meat, were purchased, chopped, minced and ground. Then, the meat (and added lard) was cooked at 70°C during 70 min in a hot water bath (cooked to the core, but not overcooked to avoid interference from the formation of genotoxic heterocyclic amines and polycyclic aromatic hydrocarbons), followed by homogenization in a food processor. After this, the 4 different meat based diets were manufactured as is documented in table 1, vacuum packed and stored at – 20°C.

2.1.2 Rat experiment

For this rat trial (ECD 14/58 (Ghent, Belgium)), 24 male Sprague-Dawley rats (± 150 g) were purchased from Janvier laboratories (France). The rats were housed in groups of 4 upon arrival and given a standard laboratory diet (Ssniff R/M-H pellets from Ssniff, Soest, Germany) and water at libitum during the first 10 days. After this adaptation period, all rats were divided ad random in 4 groups and housed individually. Then, during 14 consecutive days, each group received a different diet (provided *ad libitum* and refreshed daily), i.e. a diet based on lean chicken (= 'low fat chicken diet' or 'LFCh'), chicken with added lard (= 'high fat chicken diet' or 'HFCh'), lean beef ((= 'low fat beef diet' or 'LFBe') or beef with added lard ((= 'high fat beef diet' or 'HFBe'). The exact composition of these meat-based diets is documented in table 1. Dietary nutrients were estimated and calculated based on the known macronutrient content of lean meat (22 % protein, 1 % fat), lard and all other utilized ingredients (as listed on the wrapping/packaging). Energy content was calculated using the Atwater system.

Following 14/15 days on the experimental diets, all rats were anesthetized with 5 % isoflurane gas and euthanized by terminal blood collection from the abdominal aorta, after which the different organs were harvested. Rats were euthanized on 6 consecutive days; one rat of each dietary treatment was sacrificed in a random order each day (a more detailed account of this experiment is provided by Van Hecke *et al.*, 2016 [12]. For this particular study, the liver, duodenal mucosae and colonic mucosae were sampled from each individual rat. Tissues were rinsed with a 0.9 % saline solution and stored in 95 % of ethanol at -80°C until further sample processing.

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted. Furthermore, the local ethical committee granted approval for this particular study (ECD 14/58 (Ghent, Belgium)).

Table 1. Composition of the diets (LFCh = low fat chicken diet, HFCh = high fat chicken diet, LFBe = low fat beef diet; HFBe = high fat beef diet).

	LFCh	HFCh	LFBe	HFBe
Ingredients				
Meat (g/kg)	650	650	650	650
Chicken muscle (g/kg)	650	550	-	-
Beef muscle (g/kg)	-	-	650	550
Lard (g/kg)	-	100	-	100
Sucrose (g/kg)	200	200	200	200
Corn starch (g/kg)	79.2	79.2	79.2	79.2
Cellulose (g/kg)	22	22	22	22
Safflower oil (g/kg)	20	20	20	20
AIN76 mineral mix (low Ca: TD.79055) (g/kg)	20	20	20	20
AIN76 vitamin mix (g/kg)	6.3	6.3	6.3	6.3
Calcium phosphate (g/kg)	1.3	1.3	1.3	1.3
Choline bitartrate (g/kg)	1.2	1.2	1.2	1.2
Calculated nutrients				
Protein (%)	13.7	11.6	13.7	11.6
Fat (%)	2.7	12.5	2.7	12.5
Carbohydrates (%)	30.1	30.1	30.1	30.1
Meat/powder diet ratio	65/35	65/35	65/35	65/35
Calculated Energy (kcal/g FM)	2.0	2.8	2.0	2.8

2.2 DNA extraction, DNA hydrolysis and DNA adduct extraction

DNA from liver tissue, duodenal mucosae and colonic mucosae was extracted by means of the NucleoSpin Tissue Machery Nagel DNA extraction kit (Machery Nagel GmbH & Co., Düren, Germany) according to the protocol described by the manufacturer. DNA concentration and purity in each sample were determined with a Nanodrop ND-1000 spectrophotometer (Isogen Lifescience, Ijsselstein, The Netherlands).

The obtained DNA in each individual sample was then subjected to an earlier reported and validated DNA adduct extraction protocol [13]. In brief, all DNA samples were hydrolyzed in

0.1 M formic acid at 80°C during 30 min Ensuing this, sample purification and cleanup was performed with solid-phase extraction (SPE) (Oasis® HLB cartridges (1cc, 30 mg) Waters (Milford, USA)), after which all eluates were evaporated to dryness under vacuum at room temperature. In the final step, all samples were suspended in 100 µL of 0.05 % of acetic acid in water and stored at -20°C until analysis.

2.3 DNA adduct analysis

2.3.1 Reagents and chemicals

Analytical standards for M_1G, CrodG and their respective internal standards; M_1G-$^{13}C_3$ and CrodG-^{13}C,$^{15}N_2$, were purchased from Toronto Research Chemicals (Toronto, Canada). O^6-MedG and O^6-d_3-Me-dG (internal standard for both O^6-MedG and O^6-CM-dG) were obtained from Sigma-Aldrich (St. Louis, USA). O^6-CMdG (O^6-carboxymethyl-2'-deoxyguanosine) was kindly provided by Dr. S. Moore from Liverpool John Moores University (UK).

O^6-CMdG, O^6-MedG, O^6-d_3-MedG, CrodG and CrodG-^{13}C,$^{15}N_2$ were hydrolyzed to their nucleobase form in 0.1 M formic acid at 80°C during 30 min. All standards were diluted in methanol and stored at -20°C in stock and working solutions of 500 ng/mL and 5 ng/mL respectively.

2.3.2 UHPLC-HRMS analysis

A robust, validated UHPLC-HRMS method [11] enabled targeted and untargeted DNA adduct analysis. Analysis was performed on a hybrid Quadrupole-Orbitrap HRAM (Q-Exactive™, Thermo Fisher Scientific, San José, USA) coupled to a HESI-II source as described by Hemeryck *et al* [11]. Internal calibration of the MS detector was performed daily by infusion of calibration mixtures prepared according to the protocol described in the operations manual (Thermo Fisher Scientific, San José, USA). Instrument control was performed with Chromeleon Xpress and Xcalibur™ 3.0.

Targeted analysis included the detection and quantification of O^6-MeG, O^6-CMG, M_1G and CroG. Untargeted DNA adduct analysis was enabled by full scan MS acquisition at 100,000 FWHM in a range of 70 to 700 Da.

2.4 Data processing

2.4.1 Xcalibur™

O^6-MeG, O^6-CMG, M_1G and CroG were identified by means of an analytical standard and quantified based on a 10-point calibration curve (5, 10, 25, 50, 100, 200, 300, 400 and 500 pg/mL). Xcalibur™ Quan enabled data processing.

2.4.2 ToxFinder™ profiling and GENE-E marker selection

The use of ToxFinder™ software (Thermo Fisher Scientific, San José, USA) and an in-house DNA adduct database [11] allowed screening of the full scan HRMS spectra of each sample for the possible presence of known diet-related DNA adducts based on m/z. The considered inclusion criteria consisted of a minimum signal intensity of 10,000; a maximum mass deviation of 10 ppm, and the presence of a C^{13} isotope. Visualization of the obtained output from ToxFinder™ data processing was enabled by GENE-E matrix (http://www.broadinstitute.org/cancer/software/GENE-E/). Hierarchical clustering of samples was performed by means of 'one minus pearsons correlation', whilst the GENE-E marker selection tool (all possible permutations) was used to search for potential markers. Student's t-test was used for statistical interpretation of DNA adduct levels in tissue samples from rats on a different diet (n = 6 per group).

2.4.3 Sieve™ chromatographic peak selection with database lookup

Sieve™ 2.1 (Thermo Fisher Scientific, San José, USA) was implemented to screen for the presence and significance of known diet-related DNA adducts in the different DNA samples. All ions eluting between 0.7 and 5.5 min (of chromatographic analysis) with an m/z value between 70 and 700 Da were retained. The maximum peak width consisted of 0.5 min and maximum mass deviation was set at 10 ppm. Positive and negative ions were selected in separate experiments. The maximum number of frames was 200,000, whilst minimal peak intensity was set at 50,000 (arbitrary units). After automated processing, Sieve™ reported the m/z value, RT and abundance of each detected ion. The database lookup function was used to putatively identify the detected and selected ions by matching the theoretical m/z values of the diet-related

DNA adducts in the in-house database to the m/z values of the detected ions. To be able to select DNA adducts that are (significantly) higher or lower in tissue from rats that received a different diet, Sieve™ pairwise comparison experiments were executed for each tissue type separately in both the negative and positive ionization mode. Tissue DNA adduct levels were compared for the HFBe and LFBe diet, the HFCh and LFCh diet, the HFBe and HFCh diet, and the LFBe and LFCh diet, resulting in 24 different Sieve™ experiments.

2.4.4 Simca™: Orthogonal Partial Least Squares Discriminant Analysis

Sieve™ was used to select all chromatographic peaks in all samples (= 3 tissue types x 24 rats) simultaneously with the exact same settings as described above (= 2 separate Simca™ experiments, 1 for positive ions, 1 for negative ions). Simca™ 13 (Umetrics AB, Umeå, Sweden) was used to enable processing of multivariate omics data by means of OPLS-DA. Automated Simca™ data modeling was performed after correction for the amount of DNA in each sample, logarithmic data transformation and Pareto scaling. R^2 was assessed to ensure goodness of fit (minimal threshold of 0.5) and Q^2 was used to assess the predictive ability of the method (minimal threshold of 0.5). Discriminative/predictive ions were selected based on their excentric position in the S-plot and a VIP score above 1. A VIP above 1 demonstrates a high influence; a VIP below 1, but above 0.8 demonstrates a moderate influence, and a VIP below 0.8 reflects a low influence.

3. RESULTS

The average initial and final body weight of the rats did not differ among the dietary treatments (a more detailed account is provided by Van Hecke *et al.,* 2016 [12]. Rats on the diets with added lard had a significantly lower feed intake (-25 %) compared to rats on the diets without added lard, but there was no difference ($p = 0.751$) in metabolizable energy intake (data published previously by Van Hecke *et al.*, 2016 [12].

Overall, several diet-related DNA adducts could be retrieved in DNA obtained from rat liver, duodenal and colonic mucosae by means of UHPLC-HRMS(/MS), which will be discussed in detail below.

3.1 O^6-CMG, O^6-MeG, M_1G and CroG DNA adduct levels (Xcalibur™ Quan)

The O^6-CMG DNA adduct could not be retrieved in any of the samples under investigation. M_1G could be detected (levels < limit of quantification) in DNA obtained from the duodenal mucosa of 1 rat on a HFBe diet, and in a DNA sample obtained from the colonic mucosa of a rat on a HFCh diet. The O^6-MeG adduct could be quantified in 6 out of 24 liver samples, 2 out of 24 duodenal samples and 3 out of 24 colon samples. The CroG DNA adduct could be detected and quantified in 1 duodenum sample, 2 liver samples and 3 colon samples. There was no clear distinction according to diet although both O^6-MeG and CroG DNA adduct levels appeared to be lower in liver tissue in comparison to duodenal and colonic mucosal tissue after correction for the amount of DNA in each sample (table 2 and 3).

Table 2. Obtained amount of DNA (ng/µL) for each sample.

Rat ID	Liver	Duodenum	Colon
1	83.04	46.36	45.70
2	98.62	69.64	100.62
3	87.96	39.40	39.04
4	55.16	39.58	20.06
5	76.76	35.76	29.56
6	89.86	72.22	28.16
7	84.76	34.16	12.38
8	64.32	31.16	29.64
9	81.86	24.66	24.92
10	69.00	51.30	35.46
11	81.82	22.26	27.10
12	94.06	35.06	32.04
13	82.90	32.24	59.46
14	64.90	43.34	24.08
15	107.8	30.44	14.04
16	74.06	34.34	9.24
17	69.40	52.34	21.06
18	80.24	43.02	42.86
19	66.28	53.80	25.62
20	81.28	37.54	38.06
21	83.54	67.72	33.32
22	76.50	34.26	19.92
23	99.04	68.18	66.16
24	75.36	37.72	45.96

Table 3. Targeted DNA adduct levels (in 100 µg of DNA) obtained from liver (L), duodenum (D) and colon (C); LFCh = low fat chicken diet, HFCh = high fat chicken diet, LFBe = low fat beef diet, HFBe = high fat beef diet; N.D. = not detected, N.Q. = detected below quantification limit.

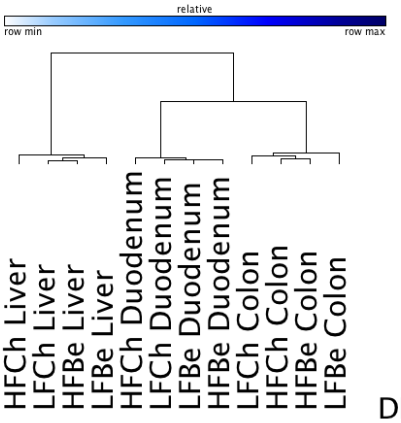
		O^6-MeG (pg/mL)			M_1G (pg/mL)			CroG (pg/mL)		
Rat ID	**Diet**	**L**	**D**	**C**	**L**	**D**	**C**	**L**	**D**	**C**
1	LFCh	**13**	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
2	HFCh	**7**	N.D.	N.D.	N.D.	N.D.	**N.Q.**	N.D.	N.D.	N.D.
3	LFBe	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
4	HFBe	**11**	**530**	**222**	N.D.	N.D.	N.D.	N.D.	N.D.	**8**
5	LFCh	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
6	HFCh	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
7	LFBe	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
8	HFBe	N.D.	N.D.	N.D.	N.D.	**N.Q.**	N.D.	N.D.	N.D.	N.D.
9	LFCh	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
10	HFCh	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
11	LFBe	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
12	HFBe	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
13	LFCh	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
14	HFCh	N.D.	N.D.	**489**	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
15	LFBe	N.D.	**539**	**230**	N.D.	N.D.	N.D.	N.D.	N.D.	**11**
16	HFBe	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
17	LFCh	**14**	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
18	HFCh	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	**2**	**25**	N.D.
19	LFBe	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
20	HFBe	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
21	LFCh	**11**	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
22	HFCh	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	**2**	N.D.	**11**
23	LFBe	**8**	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
24	HFBe	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

3.2 ToxFinder™ DNA adduct profiling

An overview of the results of ToxFinder™ data processing and GENE-E clustering (pearsons correlation) is presented in the heat map (figure 1). Only DNA adduct types that could be retrieved in a vast majority of DNA samples (present in ≥ 4 out of 6 samples) were included in the heat map.

Next page:

Figure 1. Heat map of average (n=6 for each sample type) DNA adduct types and levels in liver, duodenal and colon DNA after correction for the amount of DNA per sample. Darker shades of blue represent higher average DNA adduct levels. HF indicates a high fat content in the diet, LF indicates a low fat content in the diet, Ch stands for chicken and Be stands for beef. RT is expressed in min, an asterix marks a different isomer of a certain DNA adduct that had already been detected at an earlier RT (different RT = different isomer), and a grey box represents the total absence of DNA adduct detection in those particular samples. The ionization mode in which each DNA adduct type was detected, is provided between brackets after each DNA adduct name.



DNA adduct	RT
Trihydroxybutyl-U (-)	0.72
Cro-G (+)	0.96
Carboxyl-A (+)	0.96
Methyl-C (+)	0.96
OHE-C (+)	1.01
1,N2-propano-G (+) of Carboxyethyl-A (+)	1.04
Hydroxy-C (+)	1.10
Nitro-C (+)	1.15
Carboxymethyl-G (-) or Glyoxal-G (-)	1.19
Carboxyethyl-G (+) or Methylglyoxal-G (+) or Carboxyhydroxyethyl-A (+)	1.24
Trihydroxybutyl-T (-)	1.25
Hydroxymethyl-G (+) or Methoxy-G+	1.32
Hydroxybutyl-A (+)	1.63
N2,3-etheno-G (+)	1.71
1,N2-propano-G* (-) or Carboxyethyl-A* (-)	1.75
Carboxyl-A* (+)	1.77
Hydroxymethyl-A (+) or Methoxy-A (+) or Methyl-G (+)	1.85
1,N2-propano-G** (+) of Carboxyethyl-A** (+)	1.93
Methoxymethyl-G (+)	2.21
M2-G (-)	3.56
M2-acetaldehyde-A (-)	3.65
M1-acetaldehyde-A (-)	3.65
Dimethyl-G (+) or Ethyl-G (+) or Hydroxyethyl-A (+) or Methoxymethyl-A (+)	3.66
Hydroxybutyl-A (+)	3.78
Hydroxyhydro-C (-)	3.79
Hydroxybutyl-G (+)	3.83
Dimethyl-G* (+) or Ethyl-G* (+) or Hydroxyethyl-A* (+) or Methoxymethyl-A* (+)	3.88
Hydroxyhydro-C* (-)	4.12
M2-acetaldehyde-A* (-)	4.18
M1-acetaldehyde-A* (-)	4.18
Dodecenoate-C (+)	4.22
Trihydroxybutyl-U (+)	4.27
Hep-G (-)	4.29
Dodecenoate-A (-)	4.35
HydroxyethylC (-) or Methoxymethyl-C (-)	4.37
Dodecenoate-G (-)	4.40
Carbamoylethyl-G (+) of Carbamoylhydroxyethyl-A (+)	4.50
HNE-C (+)	4.61
M3-C (+)	4.64
Hydroxyethyl-C* (-) or Methoxymethyl-C* (-)	4.80
Dodecenoate-A (+)	4.86
Dodecenoate-A* (+)	4.94
Oct-G (-)	5.35
Carbamoylhydroxyethyl-G (+)	5.48
M2-acetaldehyde-G (-)	5.50

3.3 Significantly higher or lower DNA adduct levels according to Sieve™ peak integration and database lookup

Different putatively identified DNA adducts could be detected in all tissue types and samples. Two sample differential analysis enabled pairwise comparison of DNA adduct levels in each tissue type according to diet. DNA adduct types that appeared to be distinctly higher in beef *vs.* chicken are of particular interest to this study due to their possible role in the unknown underlying pathways that causally link red meat consumption and CRC. The same applies for DNA adducts that appear to be higher upon digestion of a 'high fat' (HF) diet *vs.* a 'low fat' (LF) diet. Therefore, only those types of DNA adducts are presented in table 4, 5 and 6, and discussed further on. All other DNA adduct types can be consulted in table 7, 8 and 9, but will not be discussed.

Table 4. Significantly higher levels ($p < 0.05$ or (*) $p < 0.10$) of putatively identified DNA adducts (relevant to hypothesis) in colonic mucosae samples after two sample differential analysis by means of Sieve™ database lookup analysis.

Higher in	DNA adduct	Charge	RT (min)	Δ ppm
HFCh *vs.* LFCh	Trimethyl-G	+	0.79	5.10
	$N^{2,3}$-etheno-G	+	2.10	4.11
	Butyl-G (*)	+	2.16	6.18
	Methoxy-A (*)	+	4.70	8.85
HFBe *vs.* LFBe	Hydroxyethyl-T (*)	+	1.01	7.19
	Carboxyethyl-T (*)	+	1.10	6.04
	Carboxymethyl-T or Carboxyethyl-U (*)	+	1.10	4.22
	M_1G (*)	+	1.15	8.86
	Methyl-C-glycol (*)	+	1.37	4.16
	Hydroxynonenal-G (*)	+	1.51	0.89
	Oxohexenal-A (*)	+	4.00	0.57
HFBe *vs.* HFCh	M_1-C (*)	-	1.36	1.92
LFBe *vs.* LFCh	1,N^6-etheno-A (*)	+	0.71	9.70
	Carboxy-T (*)	-	0.72	2.42
	M_1-C (*)	-	1.42	2.66
	U-glycol (*)	-	1.89	4.38
	T-glycol (*)	+	2.16	3.60
	Formamidopyrimidine-A	+	4.86	8.05
	Hydroxynonenal-G	+	5.34	4.08

Table 5. Significantly higher levels ($p < 0.05$ or (*) $p < 0.10$) of putatively identified DNA adducts (relevant to hypothesis) in duodenal mucosae samples after two sample differential analysis by means of Sieve™ database lookup analysis.

Higher in	DNA adduct	Charge	RT (min)	Δ ppm
HFCh *vs.* LFCh	Glyoxal-G	-	1.17	0.69
	Hydroxy-A	+	1.35	3.33
	Heptenaletheno-C	+	2.43	9.46
	Methoxymethyl-G or Hydroxyethyl-G	+	2.77	7.62
	M_2-acetaldehyde-A	+	2.83	5.52
	Dihydro-T (*)	+	3.97	3.99
HFBe *vs.* LFBe	Crotonaldehyde-G	+	0.99	0.71
	Octenal-G (*)	+	4.33	6.01
HFBe *vs.* HFCh	Carboxyethyl-T	+	0.71	5.42
	Trimethyl-G (*)	-	0.86	8.40
	Pentenal-G	-	0.94	4.71
	Crotonaldehyde-G	+	0.96	0.71
	Heptenaletheno-C	+	0.99	2.39
	Oxohexenal-A (*)	+	1.04	5.25
	Hydroxybutyl-C (*)	+	1.07	3.29
	Dimethyl-T or Ethyl-T	+	1.40	7.91
	Nitro-U	+	2.04	2.16
	Butyl-G	+	2.18	6.55
	Oxohexenal-G	+	2.38	6.31
	Butyl-G (*)	+	3.63	6.62
	M_2-acetaldehyde-A (*)	-	4.18	2.78
	Oxohexenal-G	+	5.03	3.89
	Oxohexenal -G (*)	+	5.50	4.50
LFBe *vs.* LFCh	$1,N^2$-propano-G (*)	-	1.04	7.17
	Hydroxyhydro-C (*)	-	4.12	4.77

Table 6. Significantly higher levels ($p < 0.05$ or (*) $p < 0.10$) of putatively identified DNA adducts (relevant to hypothesis) in liver samples after two sample differential analysis by means of Sieve™ database lookup analysis.

Higher in	DNA adduct	Charge	RT (min)	Δ ppm
HFCh *vs.* LFCh	Methoxy-A (*)	-	1.5	2.86
	M_1-acetaldehyde-G (*)	+	2.94	5.36
	Methoxy-U	+	3.52	3.45
	Nitro-T (*)	-	3.79	9.44
	Hydroxybutyl-C (*)	-	4.29	6.63
	M_1-acetaldehyde-G	-	4.85	5.97
	Pentenal-G (*)	+	5.11	1.38
HFBe *vs.* LFBe	Carbamoylhydroxyethyl-G	+	0.82	1.86
	Formyl-U (*)	+	1.29	2.15
	Methyl-C (*)	+	1.49	3.48
	M_1-C	-	3.01	3.77
	Carboxymethyl-C	+	3.88	2.51
	Heptenal-G	+	3.94	1.15
	Pentenal-G	+	5.31	4.75
	Trihydroxybutyl-T	+	5.33	8.49
HFBe *vs.* HFCh	Butyl-G (*)	+	0.71	5.00
	Formamidopyrimidine-A (*)	+	5.00	9.25
	Oxohexenal-C (*)	+	5.11	0.51
LFBe *vs.* LFCh	Methoxy-U (*)	+	1.21	2.37
	Carboxymethyl-T or Carboxyethyl-U	+	1.35	1.72
	Heptenaletheno-A (*)	-	1.39	3.22
	Heptenaletheno-A	+	4.11	4.10

Table 7. Significantly higher levels ($p < 0.05$ or (*) $p < 0.10$) of putatively identified DNA adducts (not relevant to current hypothesis) in liver samples after two sample differential analysis by means of Sieve™ database lookup analysis.

Higher in	DNA adduct	Charge	RT (min)
LFCh *vs.* HFCh	Nitro-A (*)	-	2.81
	Malondialdehyde-acetaldehyde2-A (*)	-	4.57
LFBe *vs.* HFBe	Hydroxybutyl-T (*)	+	0.93
	MethoxymethylG or HydroxyethylG (*)	+	1.49
	Nitro-A (*)	+	1.6
	Hydroxybutyl-A	+	3.69
	Butyl-G (*)	+	3.75
	Hydroxyhydro-C (*)	-	3.79
	Hydroxybutyl-G	+	3.8
	Hydroxybutyl-G (*)	-	3.84
	Octenal-G (*)	+	5.39
HFCh *vs.* HFBe	/		
LFCh *vs.* LFBe	Carboxymethyl-T or Carboxyethyl-U (*)	-	0.75
	Carbamoylethyl-G	+	0.76
	Dimethyl-A or Ethyl-A (*)	+	0.76
	Carbamoylhydroxyethyl-G (*)	+	0.82
	M3-C (*)	+	1.93
	Carbamoylethyl-G (*)	-	2.17
	Oxohexenal-A (*)	+	3.88

Table 8. Significantly higher levels ($p < 0.05$ or (*) $p < 0.10$) of putatively identified DNA adducts (not relevant to current hypothesis) in duodenal mucosae samples after two sample differential analysis by means of Sieve™ database lookup analysis.

Higher in	DNA adduct	Charge	RT (min)
LFCh *vs.* HFCh	Hydroxyethyl-T	+	0.71
	Hydroxyethyl-A	+	0.74
	Butyl-G	+	0.96
	Octenal-G (*)	-	5.41
LFBe *vs.* HFBe	Heptenaletheno-G	-	4.79
HFCh *vs.* HFBe	Glyoxal-G	-	1.17
	Trihydroxybutyl-G (*)	+	1.96
	Heptenaletheno-C	+	3.88
	Hydroxybutyl-G (*)	-	4.09
LFCh *vs.* LFBe	Glyoxal-G (*)	-	1.14

Table 9. Significantly higher levels ($p < 0.05$ or (*) $p < 0.10$) of putatively identified DNA adducts (not relevant to current hypothesis) in colonic mucosae samples after two sample differential analysis by means of Sieve™ database lookup analysis.

Higher in	DNA adduct	Charge	RT (min)
LFCh *vs.* HFCh	Hydroxydihydro-T (*)	-	1.22
	Carboxyl-A	+	1.65
	Hydroxydihydro-T	-	2.14
	Heptenaletheno-G	+	4.06
	Methyl-C-glycol	+	4.22
	Hydroxyethyl-A	+	4.86
	Hydroxy-A (*)	+	4.92
	Carboxy-T	-	5.30
LFBe *vs.* HFBe	Heptenaletheno-A (*)	-	5.35
HFCh *vs.* HFBe	Heptenaletheno-A	-	4.85
LFCh *vs.* LFBe	Dimethyl-T or Ethyl-T	-	0.72
	Methoxy-C	-	0.89
	M2-G (*)	+	1.10
	Heptenaletheno-A (*)	+	4.45
	Hydroxybutyl-A (*)	-	5.46

3.3.1 Influence of fat content in the diet

Different putative DNA adduct types appeared to be higher in rats on a HF diet compared to a LF diet, as is documented in tables 4 to 6. None of the DNA adduct types that were significantly ($p < 0.05$) or borderline significantly higher ($p < 0.10$) in one of the three tissue types under investigation also appeared to be (borderline) significantly higher ($p < 0.10$) in one of the other two tissue types.

3.3.2 Effect of beef *vs.* chicken meat digestion

Digestion of a beef diet resulted in a significantly different DNA adduct profile in liver, duodenum and colon (see tables 4 to 6). The formyl-U DNA adduct (RT of approximately 1.90 min) was significantly higher in both liver ($p < 0.05$) and duodenal ($p < 0.10$) DNA. A significantly higher level ($p < 0.10$) of both methyl-C (RT of approximately 1.96) and dimethyl-A or ethyl-A (RT of approximately 4.86) could be retrieved in the small and large bowel.

3.4 Discriminating DNA adducts (Simca™ modeling)

An acceptable OPLS-DA model that enables a clear distinction between samples from rats in different dietary groups could not always be constructed. DNA adducts in colon and liver samples could not be modeled according to diet, although samples from different tissue types could easily be distinguished and modeled at all times (figure 2 & 3). DNA adducts in duodenal DNA could be modeled under certain conditions; the effect of the HFBe diet could be modeled *vs.* the HFCh diet in positive ionization mode. The same applies for samples from rats on a HFCh diet *vs.* rats on a LFCh diet. In negative ionization mode, the beef diet could be modeled *vs.* the chicken diet regardless of fat content, whilst the HFBe diet *vs.* the LFBe diet could be modeled with the retained negative ions as well. Different putative DNA adducts demonstrated a high VIP score (> 1) and an excentric position in the corresponding S-plot (S-plots of valid models are provided in figures 4 to 8). Table 10 provides an overview of the potentially discriminating DNA adducts of interest (that could be retrieved with Simca™). When calculating the true positive (= sensitivity) and true negative rate (= specificity) of the potential discriminants, none of the retrieved DNA adduct types allowed a perfect discrimination according to the consumption of a specific meat type.

3.5 Selection of DNA adduct types relevant to the proposed red meat hypotheses

Table 11 contains a selection of the obtained ToxFinder™, GENE-E, Sieve™ and Simca™ output. DNA adduct types were selected if they were retrieved with ToxFinder™ and (1) demonstrated significantly higher or lower levels (significant if $p < 0.05$ or borderline significant if $p < 0.10$) for a certain diet according to the student's t-test or (2) demonstrated significantly higher or lower levels (significant if $p < 0.05$ or borderline significant if $p < 0.10$) for a certain diet according to Sieve™ or (3) were singled out as a potential marker by the GENE-E marker selection tool or (4) were singled out as a potential marker by Simca™. All listed DNA adduct types could be retrieved in liver, duodenum as well as colon DNA (except for M_2G (RT 3.66), which could not be detected in liver DNA).

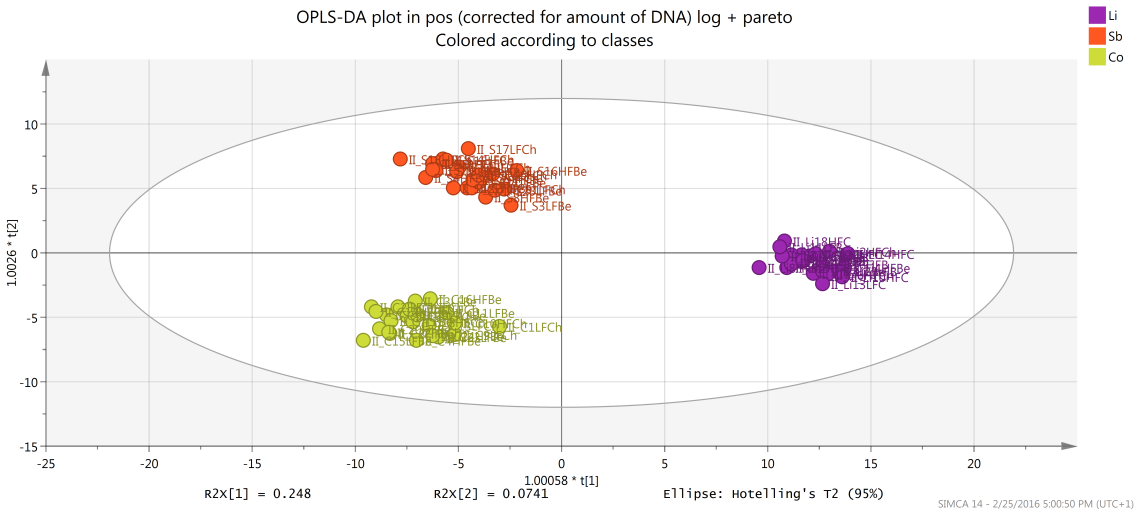


Figure 2. OPLS-DA scatter plot demonstrating a clear grouping of samples according to (the retrieved DNA adduct profile in each) tissue type in positive ionization mode; Li = liver samples (purple), Sb = duodenum samples (orange), Co = colon samples (yellow).

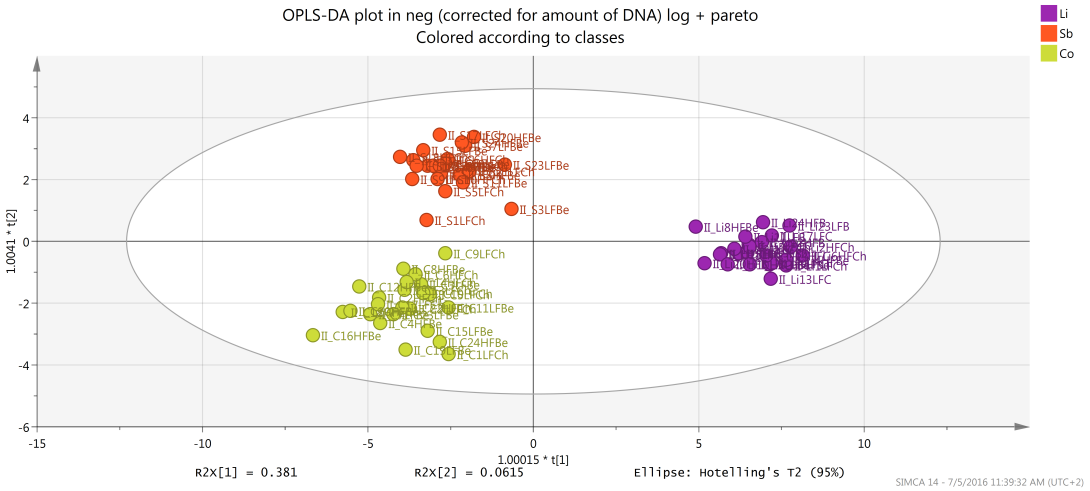


Figure 3. OPLS-DA scatter plot demonstrating a clear grouping of samples according to (the retrieved DNA adduct profile in each) tissue type in positive negative ionization mode; Li = liver samples (purple), Sb = duodenum samples (orange), Co = colon samples (yellow).

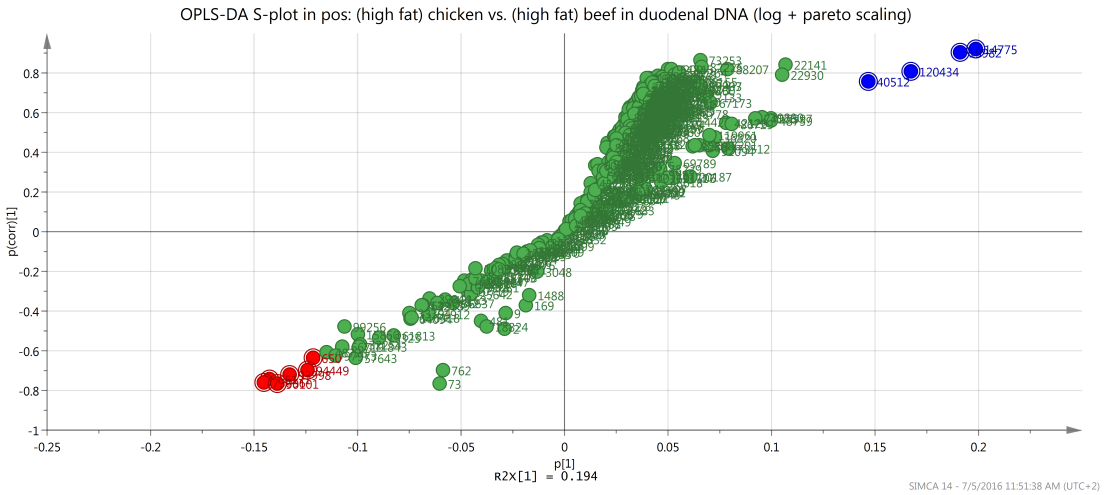


Figure 4. Valid OPLS-DA model S-plot with highlighted potential discriminating markers for high fat chicken (lower left quadrant; red) *vs.* high fat beef (upper right quadrant, blue).

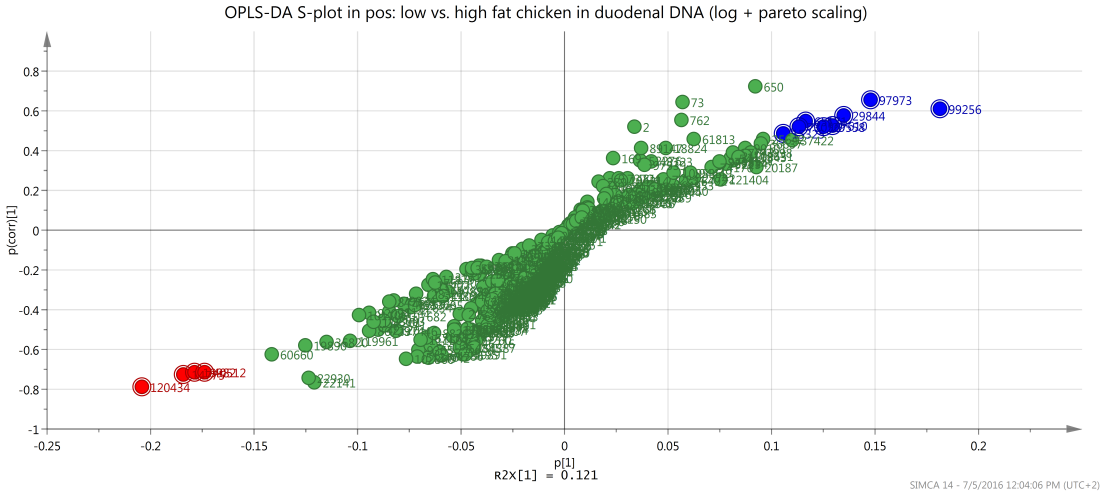


Figure 5. Valid OPLS-DA model S-plot with highlighted potential discriminating markers for low fat chicken (lower left quadrant; red) *vs.* high fat chicken (upper right quadrant, blue).

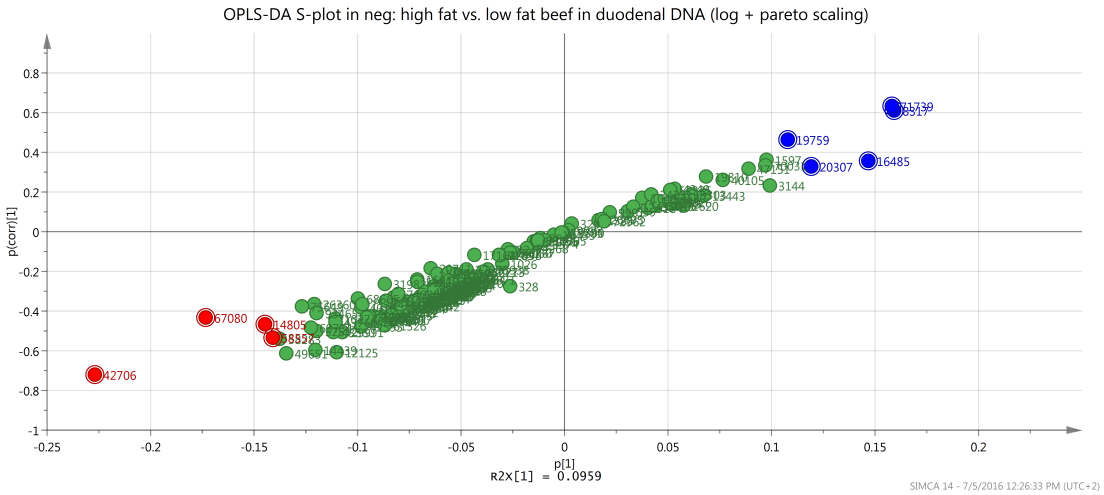


Figure 6. Valid OPLS-DA model S-plot with highlighted potential discriminating markers for high fat beef (lower left quadrant; red) *vs.* low fat beef (upper right quadrant, blue).

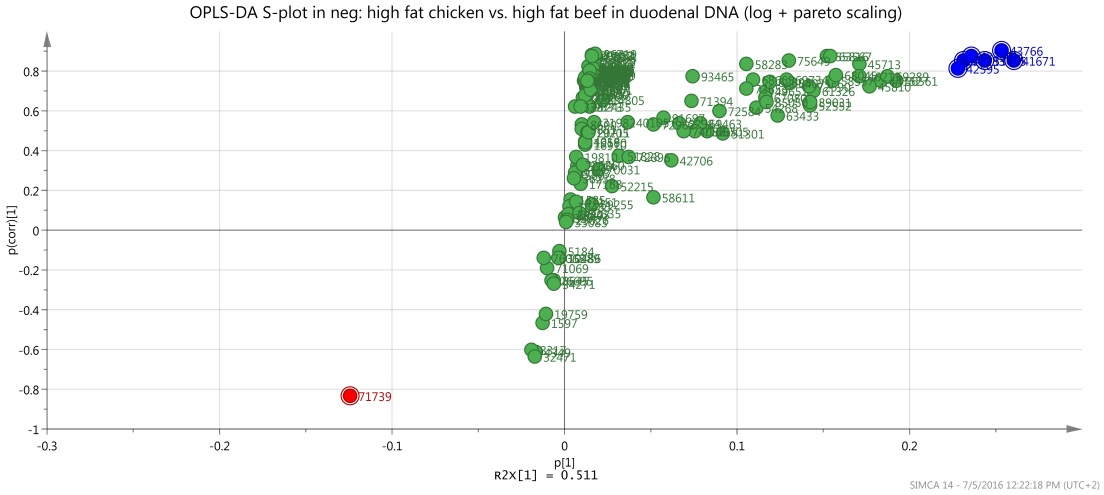


Figure 7. Valid OPLS-DA model S-plot with highlighted potential discriminating markers for high fat chicken (lower left quadrant; red) *vs.* high fat beef (upper right quadrant, blue).

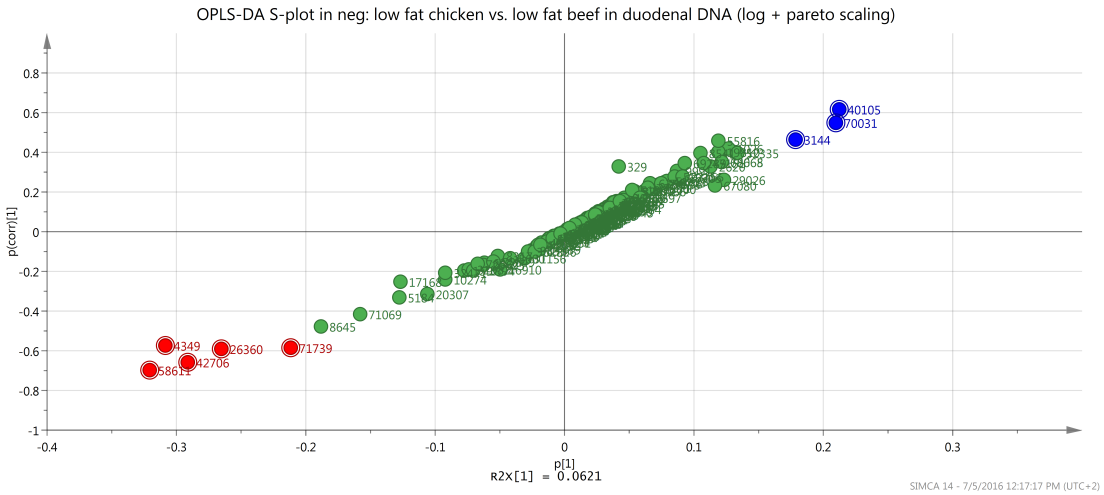


Figure 8. Valid OPLS-DA model S-plot with highlighted potential discriminating markers for low fat chicken (lower left quadrant; red) *vs.* low fat beef (upper right quadrant, blue).

Table 10. Potentially discriminating DNA adducts (with high VIP scores and an excentric S-plot position (Be = beef, Ch = chicken, HF = high fat, LF = low fat)) (Simca™ data).

DNA adduct of interest	DNA adduct type	Charge	RT (min)	Δ ppm	Potentially discriminating marker for	VIP score	Sensitivity (%)	Specificity (%)
M_2G	Lipid peroxidation	-	0.72	5.78	LF**Be** (*vs.* LFCh)	1.45	100	0
Hydroxy-A	Oxidation	-	0.89	3.33	**LF**Be (*vs.* HFBe)	1.21	100	0
Trihydroxybutyl-U	Alkylation & oxidation	+	0.96	0.12	HF**Ch** (*vs.* HFBe)	2.43	83	83
M_2G	Lipid peroxidation	+	1.32	7.67	HF**Ch** (*vs.* HFBe)	2.27	100	50
Trihydroxybutyl-T	Alkylation & oxidation	-	1.39	4.91	LF**Be** (*vs.* LFCh)	1.95	83	0
M_2-acetaldehyde-A	Lipid peroxidation	-	1.39	7.98	**LF**Be (*vs.* HFBe)	1.50	83	33
M_2-acetaldehyde-G	Lipid peroxidation	+	3.86	8.06	HF**Ch** (*vs.* HFBe)	2.65	100	67
					HFCh (*vs.* LFCh)	1.99	100	33

Table 11. Selection of DNA adduct types that could be relevant to the proposed red meat hypotheses (Be = beef, Ch = chicken, HF = high fat, LF = low fat).

DNA adduct of interest	DNA adduct type	Charge	RT (min)	Δ ppm	Selected as discriminant marker for	Selected in	Selected by	Associated p-value
Trihydroxybutyl-U	Alkylation & oxidation	-	0.72	3.21	HF**Be** *vs.* HFCh	Duodenum	Student's t-test	< 0.01
Carboxyl-A	Alkylation	+	0.96	1.50	HF**Be** *vs.* HFCh	Duodenum	Student's t-test	0.02
					HFBe *vs.* LFBe	Duodenum	Student's t-test	0.09
Crotonaldehyde-G	Lipid peroxidation	+	0.96	0.71	**HF**Be *vs.* LFBe	Duodenum	Sieve™ pairwise comparison	0.03
					HF**Be** *vs.* HFCh	Duodenum	Sieve™ pairwise comparison	0.01
				1.82	**HF**Be *vs.* LFBe	Duodenum	Student's t-test	0.01
					HF**Be** *vs.* HFCh	Duodenum	Student's t-test	< 0.01
Methyl-C	Alkylation	+	0.96	0.08	(LF & HF) **Be** *vs.* Ch	All tissues	GENE-E	0.10
Oxohexenal-C	Lipid peroxidation	+	1.01	0.23	HF**Be** *vs.* HFCh	Liver	Student's t-test	0.09
1,N_2-propano-G	Lipid peroxidation	-	1.04	7.17	LF**Be** *vs.* LFCh	Duodenum	Sieve™ pairwise comparison	0.10
Nitro-C	Nitrosation	+	1.15	0.59	HF**Be** *vs.* HFCh	Duodenum	Student's t-test	0.09
Carboxymethyl-G or Glyoxal-G	Alkylation and/or lipid peroxidation	-	1.19	4.05	HF**Be** *vs.* HFCh	Liver	Student's t-test	0.04
					HFCh *vs.* LFCh	Colon	Student's t-test	0.02
Carboxyethyl-G or carboxyhydroxyethyl-A or methylglyoxal-G	Alkylation and/or lipid peroxidation	+	1.24	1.91	HF**Be** *vs.* HFCh	Liver	Student's t-test	0.02
					HFBe *vs.* LFBe	Liver	Student's t-test	< 0.01
					HFCh *vs.* LFCh	Colon	Student's t-test	0.06
Hydroxybutyl-A	Alkylation & oxidation	+	1.63	4.89	**HF**Be *vs.* LFBe	Liver	Student's t-test	0.03
Carboxyl-A	Alkylation	+	1.85	0.48	HF**Be** *vs.* HFCh	Colon	Student's t-test	0.09

Table 11 continued.

DNA adduct of interest	DNA adduct type	Charge	RT (min)	Δ ppm	Selected as discriminant marker for	Selected in	Selected by	Associated p-value
Hydroxymethyl-A or methyl-G or methoxy-A	Alkylation & oxidation	+	1.85	0.54	**HF**Be *vs.* LFBe	Liver	Student's t-test	0.03
M_2-acetaldehyde-A	Lipid peroxidation	-	3.66	4.95	HF**Be** *vs.* HFCh	Duodenum	Student's t-test	< 0.01
M_2G	Lipid peroxidation	-	3.66	1.65	(LF & HF) **Be** *vs.* Ch	Colon & duodenum	GENE-E	0.02
					LF**Be** *vs.* LFCh	Duodenum	Student's t-test	0.03
Hydroxyhydro-C	Oxidation	-	3.82	0.71	HF**Be** *vs.* HFCh	Duodenum	Student's t-test	0.01
Hydroxybutyl-G	Alkylation & oxidation	+	3.89	4.33	**HF**Ch *vs.* LFCh	Duodenum	Student's t-test	0.04
Hydroxyhydro-C	Oxidation	-	4.12	4.77	LF**Be** *vs.* LFCh	Duodenum	Sieve™ pairwise comparison	0.08
				0.41	LF**Be** *vs.* LFCh	Duodenum	Student's t-test	0.03
M_2-acetaldehyde-A	Lipid peroxidation	-	4.18	2.78	HF**Be** *vs.* HFCh	Duodenum	Sieve™ pairwise comparison	0.10
Heptenal-G	Lipid peroxidation	-	4.29	4.89	HF**Be** vs. HFCh	Liver	Student's t-test	0.03
					LF**Be** *vs.* LFCh	Liver	Student's t-test	0.09
Hydroxyethyl-C or methoxymethyl-C	Alkylation & oxidation	-	4.38	1.45	HF**Be** *vs.* HFCh	Duodenum	Student's t-test	0.09
Carbamoylhydroxyethyl-G	Alkylation & oxidation	+	5.48	0.68	HF**Be** *vs.* HFCh	Duodenum	Student's t-test	0.04
M_2-acetaldehyde-G	Lipid peroxidation	-	5.50	3.71	**HF**Be *vs.* LFBe	Liver	Student's t-test	0.05

4. DISCUSSION

Red meat and animal fat intake related genotoxicity were assessed in male Sprague-Dawley rats. The red meat associated formation of a limited number of diet-related DNA adduct types (e.g. hydroxyguanine (an oxidative DNA lesion) and O^6-MeG (alkylation DNA adduct)) has been previously investigated in a small number of rodent and human studies [14-16]. However, up to date, there are no (published) untargeted *in vivo* DNA adductomics studies that investigate the possible genotoxic effects of the consumption of red *vs.* white meat (with or without added lard), rendering this study to be the first in its kind.

4.1 Liver *vs.* duodenum *vs.* colon

The described untargeted application of applied UHPLC-HRMS method enabled extensive DNA adduct profiling. As could be expected due to differences in tissue composition and physiology, and also digestion and metabolisation related differences in exposure to (different levels of) diet-related toxins, a different DNA adduct profile could be retrieved in each tissue type. Simca™ analysis allowed modeling of DNA adduct types and levels according to tissue type. In accordance, GENE-E demonstrated a clear clustering of liver samples, duodenal samples and colon samples, also distinctly clustering all bowel samples and thus reflecting a larger resemblance in the obtained DNA adduct profile in duodenum and colon DNA *vs.* liver DNA. DNA adduct types that are higher in colonic DNA could be relevant to the red meat CRC-hypothesis due to the fact that red meat consumption has primarily been linked to the development of cancer of the colon but not liver and/or duodenum. Hence, DNA adduct types that are higher in colon *vs.* duodenum or liver may reveal important clues on the underlying mechanism. Unfortunately, the relevance (rate of (potential) mutagenic and carcinogenic actions) of the retrieved DNA adduct types cannot be compared objectively at the time being; i.e. some DNA adduct types are highly mutagenic, some are repaired spontaneously, and some occur endogenously whilst others are or do not [8]. Therefore, the DNA adduct types that were distinctly higher or lower in liver, duodenum and/or colon were not explored further in this chapter.

4.2 The effect of meat type and fat content in the diet on the DNA adductome

Differences in DNA adduct levels according to diet were investigated for each tissue type separately. Three out of four targeted DNA adducts could be detected (O^6-MeG, CroG and M_1G) in rat liver, duodenum and colon. Due to the fact that the total amount of DNA in each sample was rather low, we were unable to confirm or refute any possible relation between O^6-MeG, M_1G or CroG levels and meat type or fat content in the diet. The fourth DNA adduct; i.e. O^6-CMG, could not be retrieved in this study although its presence has previously been reported in both rats and humans [8, 14, 17]. In the current study, the amount of DNA in the samples could have been too low to detect O^6-CMG although the detection limit of the utilized method may have been a limiting factor as well [11]. Alternatively, since rats do not possess an enterosalivary cycle of nitrate and the meat diets did not contain relevant amounts of nitrite, the formation of O^6-CMG adducts *via* the formation of the hypothesized NOC precursors could have been negligible [18]. Nevertheless, in future studies, the amount of DNA per sample should be increased (≥ 100 μg) to be able to thoroughly assess the influence of a specific diet on the presence and levels of O^6-MeG, O^6-CMG, CroG and M_1G DNA adducts. Even more so since previous research has already pointed out their potential *in vivo* relevance in relation to diet [7, 8, 14, 16, 19].

Extensive data processing by means of different omics software packages enabled us to single out 22 different putative DNA adduct types that were higher in rat colon, duodenum or liver after digestion of a beef based diet (compared to a diet with chicken) and/or a HF diet (compared to a LF diet); 14 DNA adduct types appeared to be significantly higher after consumption of beef (*vs.* consumption of chicken), 3 DNA adduct types demonstrated an increase upon daily HF consumption (*vs.* LF), and 5 DNA adducts demonstrated an increase after the consumption of a diet with a high fat content (compared to the corresponding LF diet) as well as a beef diet (compared to the consumption of a chicken diet). Since all 22 selected DNA adduct types originate from alkylation, nitrosation and/or oxidation processes, a more in-depth investigation of the retrieved DNA adduct types is highly relevant to the red meat-CRC hypothesis; i.e. NOCs and LPOs rise upon red meat and high fat intake, and are prone to form DNA adducts [4, 8].

It should be noted that low fat and high fat meat diets slightly differed in macronutrient as well as energy content. This may have influenced DNA adduct levels, but was inherent to the experimental setup, and thus unavoidable. The addition of lard to, and the simultaneous reduction of meat in high fat diets directly resulted in an increased fat and energy content, whilst also reducing protein and water content in high fat meat diets compared to low fat meat diets. Shifts in the DNA adductome due to the digestion of a high fat meat diet in this study may thus alternatively be related to a higher energy and/or lower protein intake. If so, the observed DNA adduct changes due to digestion of high *vs.* low fat meat diets may not be relevant to the red (and processed) meat CRC hypothesis since previous research has not been able to link total energy and protein intake to CRC risk [20, 21]. All of the above does however not interfere with the correct interpretation of DNA adductomic differences due to red *vs.* white meat digestion since, in order to assess red *vs.* white meat genotoxicity, the effect of a low fat beef diet was only compared to that of a low fat chicken diet, whilst the same applies for high fat diets.

4.3 DNA adducts that increased after the daily consumption of beef

Trihydroxybutyl-U (RT of 0.72), carboxyl-A (RT of 1.85 min), methyl-C, oxohexenal-C, 1,N_2-propano-G, nitro-C, M_2-acetaldehyde-A (observed at two different RTs), M_2G (RT of 3.66 min), hydroxyhydro-C (observed at two different RTs), heptenal-G, hydroxyethyl-C (or methoxymethyl-C) and carbamoylhydroxyethyl-G DNA adducts appeared to be higher in beef fed rats compared to chicken fed rats.

Of these DNA adduct types, only methyl-C, M_2G and carboxyl-A demonstrate a significant increase in colon DNA, the major tissue type of interest. Methylation of C at its 5th carbon atom induces the formation of 5-methyl-C. 5-Methyl-C, also known as the '6th base' of the mammalian genome, plays an important role in gene expression, genomic imprinting and suppression of transposable elements, and is therefore of specific interest in the field of epigenetics [22]. However, the methyl group in the methyl-C compound that could be detected in this study (and was singled out as a discriminative DNA adduct type for all tissue types according to GENE-E), may be positioned elsewhere, which means the retrieved methyl-C molecule could just as well correspond to N^3-methyl-C, N^4-methyl-C or O^2-methyl-C [23]. Direct or indirect addition of a carboxyl group to the adenine nucleobase is an unspecific reaction type that cannot easily be linked to a specific precursor. The gut microbiome may be able to induce the formation of

carboxyl-A directly or indirectly (e.g. *via* NOC formation) although this is merely speculative. Carboxyl-A has not been studied extensively but its C analogue (carboxyl-C) has been detected *in vivo* (genomic DNA of mouse embryonic stem cells and mouse organs) and has been linked to the enzyme mediated demethylation of 5-methyl-C [22]. Since demethylation of methyl-A has been described as well (=DNA repair pathway) [24], the mode of action may be similar, possibly resulting in the *in vivo* formation of carboxyl-A. The major MDA DNA adduct is an important LPO) is the monomeric M_1G DNA adduct although multimeric DNA adducts - like the putatively detected M_2G - can also be formed after polymerization of 2 or more MDA molecules. The formation of multimeric MDA DNA adducts does not occur as fast or frequent as monomeric MDA DNA adduct formation under physiological conditions and is relatively slow at neutral pH. However, according to Marnett *et al.* [7], multimeric MDA DNA adduct types can occur in certain - unspecified - *in vivo* circumstances.

Oxohexenal-C and heptenal-G appeared to be significantly higher in liver DNA after daily beef consumption, and for heptenal-G, the effect was observed for both the LF and HF beef diet. Oxohexenal and heptenal are products of lipid peroxidation. Shorter chain LPOs like acrolein and crotonaldehyde are more reactive towards biomacromolecules than the longer chain LPOs like heptenal and oxohexenal, but acrolein and crotonaldehyde primarily originate from ω-3 fatty acids, whilst the longer chain enals appear to originate from ω-6 fatty acids exclusively [25]. Since dietary ω-6 polyunsaturated fatty acids have been linked to colon tumorigenesis in F344 rats [26], the retrieval of oxohexenal-C and heptenal-G could be of particular interest.

The tentatively identified trihydroxybutyl-U, $1,N_2$-propano-G, nitro-C, M_2-acetaldehyde-A, hydroxyhydro-C, hydroxyethyl-C (or methoxymethyl-C) and carbamoylhydroxyethyl-G DNA adducts were exposed as discriminating DNA adducts for beef *vs.* chicken digestion in duodenal DNA. The trihydroxybutyl-U molecule has hardly been studied and could just as well correspond to an isomer with the exact same mass (like e.g. trihydroxypropyl-T). Both $1,N_2$-propano-G and M_2-acetaldehyde-A are products of lipid peroxidation derived DNA adduct formation. $1,N_2$-propano-G is formed due to exposure of G to acrolein [25], an ubiquitous and highly reactive LPO, while M_2-acetaldehyde-A can be derived from the interaction of A and a malondialdehyde-acetaldehyde conjugate consisting of 2 MDA molecules and 1 acetaldehyde molecule [27]. The possible *in vivo* formation of M_2-acetaldehyde protein adducts was confirmed by Tuma *et al.* [28], although this might be the first time that the *in vivo* formation of (2 different) M_2-acetaldehyde DNA adducts is suggested. Nitro-C, hydroxyhydro-C, hydroxyethyl-C (or

methoxymethyl-C) and carbamoylhydroxyethyl-G are formed upon nitrosation, oxidation and/or alkylation of C and G, which are very unspecific ways of DNA adduct formation that cannot easily be retraced to its exact origin/precursor. At the time, more detailed information on nitro-C is not available since it appears that nitro-C has hardly been studied. The available information on hydroxyhydro-C is scarce as well, although this compound has previously been identified in mammalian DNA [29]. To the best of our knowledge, hydroxyethyl-C and/or methoxymethyl-C have never been detected *in vivo*. Carbamoylhydroxyethyl-G formation on the other hand, has been linked to exposure to acrylamide (used in industry, present in cigarettes and foods processed at high temperatures) in rats although acrylamide exposure in this study is not very likely [30]. However, nothing in its chemical structure suggests that carbamoylhydroxyethyl-G formation is strictly limited to the occurrence of acrylamide exposure since the added chemical group is not highly specific; i.e. carbamoylation and alkylation of macromolecules can also be induced by e.g. nitrosourea compounds (a specific group of NOCs) [31]. The latter hypothesis is far more likely following the consumption of beef and the subsequent increased exposure to the NOC formation promoting heme molecule [4].

4.4 DNA adducts that increased due to the intake of a high fat diet

Hydroxybutyl-A, hydroxymethyl-A (or methyl-G or methoxy-A) and hydroxybutyl-G significantly increased in liver or duodenal DNA after the daily consumption of a HF meat diet. Airoldi *et al.* [32] already documented the *in vivo* formation of hydroxybutyl-G in urothelial and hepatic DNA after administration of a single dose of N-nitrosobutyl(4-hydroxybutyl)amine, a carcinogenic NOC, to rats. A similar mechanism may likewise be applicable for hydroxybutyl-A, although additional research is required to support this statement and confirm the detection of hydroxybutyl-A in rat liver DNA. The mass that corresponds to hydroxymethyl-A, methyl-G or methoxy-A could not be identified as O^6-MeG or N^7-methyl-G by means of analytical standards (O^6-MeG elutes at 2.83 min [11] and N^7-methyl-G elutes at 1.50 min (unpublished data)). According to literature, N^7-methyl-G is the predominantly formed methyl-G isomer, whilst O^6-MeG occurs far less frequently (e.g. 400 N^7-methyl-G molecules compared to 1 O^6-MeG molecule by the S-adenosylmethionine enzyme, a methyl group donor that contributes to physiological DNA methylation) [8]. Alternative options for identification include N^1-, and N^3-methyl-G [33] or a methoxy-A [34] or hydroxymethyl-A [35] isomer.

4.5 DNA adducts associated with the intake of a beef as well as a high fat diet

The putatively identified carboxyl-A (RT of 0.96 min), CroG (RT 0.96 min), carboxymethyl-G (or glyoxal-G), carboxyethyl-G (or carboxyhydroxyethyl-A or methylglyoxal-G) and M_2-acetaldehyde-A (RT of 5.50 min) were higher in rat DNA after digestion of a beef diet as well as a meat diet with added fat.

In colon DNA, an increase of carboxymethyl-G (or glyoxal-G) and carboxyethyl-G (or carboxyhydroxyethyl-A or methylglyoxal-G) occurred due to the consumption of a HF (chicken) diet (in comparison to a LFCh diet). The carboxymethyl-G compound could not be identified as O^6-CMG since it did not co-elute with an O^6-CMG standard (which has a RT of 1.54 min [11]). However, two very likely alternatives are N^7-carboxymethyl-G or glyoxal-G for the reason that (a) nitrosated bile salts predominantly form N^7-carboxymethyl-G during reaction with DNA [8], and (b) glyoxal-G formation by NOCs has been documented previously [36], and (c) the glyoxal molecule, an endogenously occurring metabolite that is formed during degradation of glucose, glycated proteins and lipid peroxidation, is known to readily react with DNA [37, 38]. The latter also applies for methylglyoxal, which is a probable precursor of the methylglyoxal-G DNA adduct; the molecule that may equally correspond to carboxyethyl-G, or carboxyhydroxyethyl-A. Regardless, N^7-carboxymethyl-G [8], glyoxal-G [36], methylglyoxal-G [39], carboxyethyl-G [40] as well as carboxyhydroxyethyl-A [41] have all been studied and detected *in vitro* and/or *in vivo* in previous DNA adduct studies, rendering all of these DNA adduct types to be valid options for tentative identification.

The carboxyethyl-G (or carboxyhydroxyethyl-A or methylglyoxal-G) and carboxymethyl-G (or glyoxal-G) DNA adducts could also be retrieved in liver DNA where they significantly increased after beef as well as high fat consumption. M_2-acetaldehyde-G also increased upon beef and high fat consumption in liver DNA, plus also demonstrated a significant increase in duodenal DNA in relation to beef consumption. As was discussed earlier on for its A analogue (M_2-acetaldehyde-A, in 4.3 DNA adducts that increased after the daily consumption of beef), M_2-acetaldehyde-G could originate from the interaction of G and a malondialdehyde-acetaldehyde conjugate, but has also never formerly been detected *in vivo*.

The remaining DNA adducts; carboxyl-A and CroG, were discriminating for beef *vs.* chicken and HF *vs.* LF beef in the rat duodenum. As was already discussed above, carboxyl-A has not

previously been detected *in vivo* although it may be of importance by analogy with carboxyl-C (in relation to methyl-A and 5-methyl-C respectively) [22]. Since CroG eluted at RT 0.96 min and not at RT 3.46 min [11], we can be certain that this compound does not match with the commercially available CroG standard that was purchased beforehand. The retrieved CroG molecule could be an isomer since CroG does occur in different configurations [42].

The identity of the putatively identified DNA adducts that were discussed above, were not confirmed by means of analytical standards, which is in part because the currently commercially available number of DNA adduct standards is limited. Nevertheless, there are several valid arguments that can be raised in support of the putative identification of all DNA adduct types; (a) DNA purity was tested and confirmed for each sample after DNA extraction from liver, duodenum and colon tissue samples, (b) DNA was hydrolyzed (in acid at high temperature) and DNA adducts were extracted by means of SPE, (c) DNA adducts were separated by means of a UHPLC method that was optimized for DNA adduct separation, and (d) Q-Exactive MS analysis allows highly accurate mass measurements. Nevertheless, the findings of this study should be validated by independent follow-up studies.

5. CONCLUSION

In the past, animal DNA adduct studies have allowed researchers to gain a more thorough understanding of the role of DNA adducts in mutation and carcinogenesis. Even today, animal model studies represent one of the best options to study the relation between dietary exposure to directly or indirectly harmful chemicals, gastro-intestinal formation of genotoxic chemicals, metabolisation (resulting in activation or inactivation) and excretion of genotoxic chemicals, related DNA adduct formation and the onset of disease. This study demonstrated that beef and high fat intake (in comparison with chicken and low fat meat intake) stimulate the formation of certain types of DNA adducts that may be relevant to the red-meat-CRC hypothesis since the DNA adduct types that were studied and retrieved in liver, duodenum and/or colon are the result of DNA alkylation, nitrosation and/or oxidation processes. Nevertheless, the exact relevance of these DNA adduct types in relation to the red meat-CRC hypothesis needs to be assessed further in follow-up studies.

REFERENCES

[1] M.C. Poirier, Chemical-induced DNA damage and human cancer risk, Nat Rev Cancer, 4 (2004) 630-637.
[2] A.G. Marroquin-Cardona, N.M. Johnson, T.D. Phillips, A.W. Hayes, Mycotoxins in a changing global environment--a review, Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association, 69 (2014) 220-230.
[3] B. Stewart, C.P. Wild, editors, World Cancer Report 2014 International Agency for Research on Cancer, WHO2014.
[4] M. Oostindjer, J. Alexander, G.V. Amdam, G. Andersen, N.S. Bryan, D. Chen, D.E. Corpet, S. De Smet, L.O. Dragsted, A. Haug, A.H. Karlsson, G. Kleter, T.M. de Kok, B. Kulseng, A.L. Milkowski, R.J. Martin, A.M. Pajari, J.E. Paulsen, J. Pickova, K. Rudi, M. Sodring, D.L. Weed, B. Egelandsdal, The role of red and processed meat in colorectal cancer development: a perspective, Meat science, 97 (2014) 583-596.
[5] W. Lijinsky, N-Nitroso compounds in the diet, Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 443 (1999) 129-138.
[6] J.H. Hotchkiss, Preformed N-nitroso compounds in foods and beverages, Cancer surveys, 8 (1989) 295-321.
[7] L.J. Marnett, Lipid peroxidation-DNA damage by malondialdehyde, Mutation research, 424 (1999) 83-95.
[8] R. De Bont, N. van Larebeke, Endogenous DNA damage in humans: a review of quantitative data, Mutagenesis, 19 (2004) 169-185.
[9] J. Vanden Bussche, L.Y. Hemeryck, T. Van Hecke, G.G. Kuhnle, F. Pasmans, S.A. Moore, T. Van de Wiele, S. De Smet, L. Vanhaecke, O(6)-carboxymethylguanine DNA adduct formation and lipid peroxidation upon in vitro gastrointestinal digestion of haem-rich meat, Molecular nutrition & food research, 58 (2014) 1883-1896.
[10] L.Y. Hemeryck, C. Rombouts, T. Van Hecke, L. Van Meulebroek, J. Vanden Bussche, S. De Smet, L. Vanhaecke, In vitro DNA adduct profiling to mechanistically link red meat consumption to colon cancer promotion, Toxicology Research, 5 (2016) 1346-1358.
[11] L.Y. Hemeryck, A.I. Decloedt, J. Vanden Bussche, K.P. Geboes, L. Vanhaecke, High resolution mass spectrometry based profiling of diet-related deoxyribonucleic acid adducts, Anal Chim Acta, 892 (2015) 123-131.
[12] T. Van Hecke, L. Jakobsen, E. Vossen, F. Guéraud, F. De Vos, F. Pierre, H.C. Bertram, S. De Smet, Short-term beef consumption promotes systemic oxidative stress, TMAO formation and inflammation in rats, and dietary fat content modulates these effects, Food & Function, accepted (2016).
[13] J. Vanden Bussche, S.A. Moore, F. Pasmans, G.G. Kuhnle, L. Vanhaecke, An approach based on ultra-high pressure liquid chromatography-tandem mass spectrometry to quantify O6-methyl and O6-carboxymethylguanine DNA adducts in intestinal cell lines, Journal of chromatography. A, 1257 (2012) 25-33.
[14] M.H. Lewin, N. Bailey, T. Bandaletova, R. Bowman, A.J. Cross, J. Pollock, D.E.G. Shuker, S.A. Bingham, Red Meat Enhances the Colonic Formation of the DNA Adduct O6-Carboxymethyl Guanine: Implications for Colorectal Cancer Risk, Cancer Research, 66 (2006) 1859-1865.
[15] R.K. Le Leu, J.M. Winter, C.T. Christophersen, G.P. Young, K.J. Humphreys, Y. Hu, S.W. Gratz, R.B. Miller, D.L. Topping, A.R. Bird, M.A. Conlon, Butyrylated starch intake can prevent red meat-induced O6-methyl-2-deoxyguanosine adducts in human rectal tissue: a randomised clinical trial, Br J Nutr, 114 (2015) 220-230.

[16] J. Winter, L. Nyskohus, G.P. Young, Y. Hu, M.A. Conlon, A.R. Bird, D.L. Topping, R.K. Le Leu, Inhibition by resistant starch of red meat-induced promutagenic adducts in mouse colon, Cancer prevention research (Philadelphia, Pa.), 4 (2011) 1920-1928.
[17] M. Terasaki, Y. Totsuka, K. Nishimura, K. Mukaisho, K.H. Chen, T. Hattori, T. Takamura-Enya, T. Sugimura, K. Wakabayashi, Detection of endogenous DNA adducts, O-carboxymethyl-2'-deoxyguanosine and 3-ethanesulfonic acid-2'-deoxycytidine, in the rat stomach after duodenal reflux, Cancer science, 99 (2008) 1741-1746.
[18] F.Z. Chenni, S. Tache, N. Naud, F. Gueraud, D.A. Hobbs, G.G. Kunhle, F.H. Pierre, D.E. Corpet, Heme-induced biomarkers associated with red meat promotion of colon cancer are not modulated by the intake of nitrite, Nutrition and cancer, 65 (2013) 227-233.
[19] E. Eder, Budiawan, D. Schuler, Crotonaldehyde: a carcinogenic and mutagenic air, water and food pollutant, Central European journal of public health, 4 Suppl (1996) 21-22.
[20] D. Demeyer, B. Mertens, S. De Smet, M. Ulens, Mechanisms Linking Colorectal Cancer to the Consumption of (Processed) Red Meat: A Review, Critical reviews in food science and nutrition, 56 (2016) 2747-2766.
[21] D.E. Corpet, Red meat and colon cancer: should we become vegetarians, or can we make meat safer?, Meat science, 89 (2011) 310-316.
[22] S. Ito, L. Shen, Q. Dai, S.C. Wu, L.B. Collins, J.A. Swenberg, C. He, Y. Zhang, Tet proteins can convert 5-methylcytosine to 5-formylcytosine and 5-carboxylcytosine, Science (New York, N.Y.), 333 (2011) 1300-1303.
[23] Y. Motorin, S. Muller, I. Behm-Ansmant, C. Branlant, Identification of modified residues in RNAs by reverse transcription-based methods, Methods in enzymology, 425 (2007) 21-53.
[24] T.J. Begley, L.D. Samson, AlkB mystery solved: oxidative demethylation of N1-methyladenine and N3-methylcytosine adducts by a direct reversal mechanism, Trends in biochemical sciences, 28 (2003) 2-5.
[25] F.L. Chung, J. Pan, S. Choudhury, R. Roy, W. Hu, M.S. Tang, Formation of trans-4-hydroxy-2-nonenal- and other enal-derived cyclic DNA adducts from omega-3 and omega-6 polyunsaturated fatty acids and their roles in DNA repair and human p53 gene mutation, Mutation research, 531 (2003) 25-36.
[26] C.V. Rao, Y. Hirose, C. Indranie, B.S. Reddy, Modulation of experimental colon tumorigenesis by types and amounts of dietary fatty acids, Cancer Res, 61 (2001) 1927-1933.
[27] D. Pluskota-Karwatka, A.J. Pawlowicz, L. Kronberg, Formation of malonaldehyde-acetaldehyde conjugate adducts in calf thymus DNA, Chemical research in toxicology, 19 (2006) 921-926.
[28] D.J. Tuma, G.M. Thiele, D. Xu, L.W. Klassen, M.F. Sorrell, Acetaldehyde and malondialdehyde react together to generate distinct protein adducts in the liver during long-term ethanol administration, Hepatology (Baltimore, Md.), 23 (1996) 872-880.
[29] M. Dizdaroglu, Oxidative damage to DNA in mammalian chromatin, Mutation research, 275 (1992) 331-342.
[30] I. Manière, T. Godard, D.R. Doerge, M.I. Churchwell, M. Guffroy, M. Laurentie, J.-M. Poul, DNA damage and DNA adduct formation in rat tissues following oral administration of acrylamide, Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 580 (2005) 119-129.
[31] G.P. Wheeler, B.J. Bowdon, R.F. Struck, Carbamoylation of amino acid, peptides, and proteins by nitrosoureas, Cancer Res, 35 (1975) 2974-2984.
[32] L. Airoldi, C. Magagnotti, M. Bonfanti, L. Chiappetta, M. Lolli, C. Medana, G. De Gregorio, R. Fanelli, Detection of O6-butyl- and O6-(4-hydroxybutyl)guanine in urothelial and hepatic DNA of rats given the bladder carcinogen N-nitrosobutyl(4-hydroxybutyl)amine, Carcinogenesis, 15 (1994) 2297-2301.

[33] M.C. Di Pietro, D. Vannoni, R. Leoncini, G. Liso, R. Guerranti, E. Marinello, Determination of urinary methylated purine pattern by high-performance liquid chromatography, Journal of chromatography. B, Biomedical sciences and applications, 751 (2001) 87-92.
[34] H. Nishio, A. Ono, A. Matsuda, T. Ueda, The synthesis and properties of oligodeoxyribonucleotides containing N6-methoxyadenine, Nucleic Acids Res, 20 (1992) 777-782.
[35] H.S. el-Khadem, R. Sindric, Synthesis of 8-(hydroxyalkyl)adenines, Carbohydrate research, 34 (1974) 203-207.
[36] R.N. Loeppky, W. Cui, P. Goelzer, M. Park, Q. Ye, Glyoxal-guanine DNA adducts: detection, stability and formation in vivo from nitrosamines, IARC Sci Publ, (1999) 155-168.
[37] E.A. Abordo, H.S. Minhas, P.J. Thornalley, Accumulation of alpha-oxoaldehydes during oxidative stress: a role in cytotoxicity, Biochemical pharmacology, 58 (1999) 641-648.
[38] N. Mistry, I. Podmore, M. Cooke, P. Butler, H. Griffiths, K. Herbert, J. Lunec, Novel monoclonal antibody recognition of oxidative DNA damage adduct, deoxycytidine-glyoxal, Laboratory investigation; a journal of technical methods and pathology, 83 (2003) 241-250.
[39] C.E. Vaca, J.A. Nilsson, J.L. Fang, R.C. Grafstrom, Formation of DNA adducts in human buccal epithelial cells exposed to acetaldehyde and methylglyoxal in vitro, Chem Biol Interact, 108 (1998) 197-208.
[40] G. Cheng, M. Wang, P.W. Villalta, S.S. Hecht, Detection of 7-(2'-Carboxyethyl)guanine but Not 7-Carboxymethylguanine in Human Liver DNA, Chemical research in toxicology, 23 (2010) 1089-1096.
[41] G. Gamboa da Costa, M.I. Churchwell, L.P. Hamilton, L.S. Von Tungeln, F.A. Beland, M.M. Marques, D.R. Doerge, DNA Adduct Formation from Acrylamide via Conversion To Glycidamide in Adult and Neonatal Mice, Chemical research in toxicology, 16 (2003) 1328-1337.
[42] S. Zhang, P.W. Villalta, M. Wang, S.S. Hecht, Analysis of crotonaldehyde- and acetaldehyde-derived 1,n(2)-propanodeoxyguanosine adducts in DNA from human tissues using liquid chromatography electrospray ionization tandem mass spectrometry, Chemical research in toxicology, 19 (2006) 1386-1392.

CHAPTER VII

Discussion
&
Future perspectives

Contains parts adapted from:

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1. MAIN RESEARCH FINDINGS AND SCIENTIFIC CONTRIBUTIONS

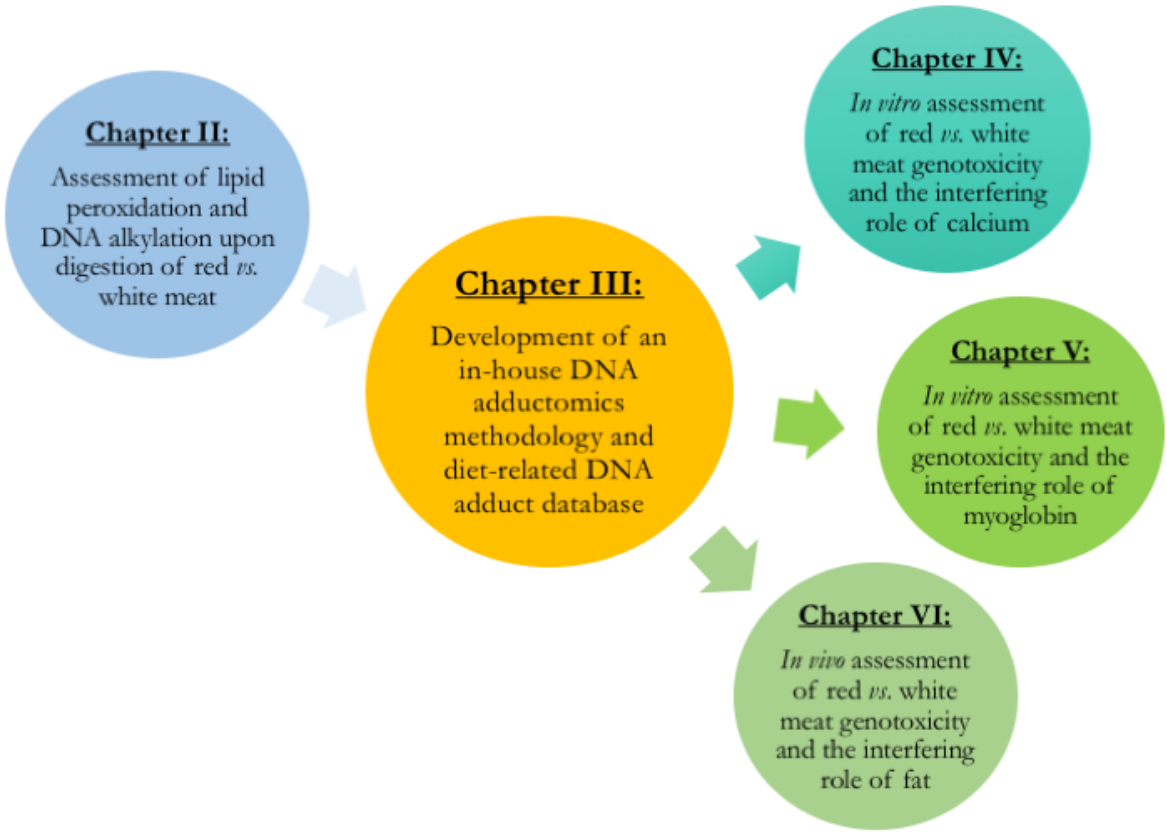

Figure 1. Schematic overview of the workflow and scientific contributions of this thesis.

1.1 Development and application of a DNA adductomics methodology to study diet-related genotoxin exposure

Exposomic studies comprise both external and internal exposure assessment in order to correctly link exposure to effect [1], which also applies to DNA adductomic studies. The most common tool used to study environmental exposure to toxins are questionnaires. However, the major flaw of questionnaires in light of exposome mapping, is the fact that they can only focus on a limited number of environmental factors and pollutants. Furthermore, questionnaires do not take unknown exposure into account, and are often subject to participant or response bias [1, 2]. Therefore, an in-depth exposure assessment requires a multi-disciplinary approach that can tackle these issues.

Focussing on the more detailed assessment of genotoxin exposure by means of analytical chemistry, two different types of studies or approaches can be distinguished; the bottom-up (targeted) and the top-down (untargeted) approach. The bottom-up approach envisions summing up all known exposure types or groups in order to characterize the exposome. The downside to this most commonly used approach is the fact that unknown exogenous and also endogenous environmental factors may be overlooked [1, 3]. The alternative strategy on the other hand; the top-down approach, reflects both known and unknown exogenous and endogenous exposures [1]. Although the latter sounds very appealing, it requires specialized untargeted "omics" technologies and methodologies, and embodies extensive data processing by means of specialized software. Nevertheless, independent experts in the field have suggested that "omics" is the present and future of (cancer) epidemiology, despite its expense and complexity [1, 4]. Therefore, application of these approaches to the field of adductomics, *via* the use of appropriately designed studies and analytical methodologies, is of high importance.

The study of DNA adduct types and levels in human tissue offers an interesting tool in several fields of research, including toxicology and cancer epidemiology. Over the years, a range of techniques and methods have been developed to study the formation of endo- and exogenous DNA adducts in different biological matrices, ranging from those based on antibodies and labeling such as immunoassays, immunohistochemistry and ^{32}P-postlabeling to advanced instrumental techniques. The latter invariably use chromatographic separation coupled with various detection methods e.g. GC-ECD, HPLC-FD, GC- or (HP)LC-MS, (LC-)NMR, and AMS [5, 6]. However, for the simultaneous detection, identification and quantification of both known and unknown DNA adducts, MS is deemed to be the most promising technique and the gold standard for DNA adduct detection [5, 7, 8].

Different research groups have explored triple quadrupole MS/MS [9, 10], ion trap MS [11, 12], TOF (time-of-flight) HRMS [13, 14] and orbitrap HRMS [15] for DNA adductomics purposes. Up until now, triple quad and ion trap technology have been applied most frequently as MS^n accommodates the need for low level DNA adduct measurements [16, 17]. However, for untargeted omics applications, HRMS is the more rational choice since accurate mass measurements simplify compound identification [16]. A relatively recent trend in MS technology is the more widely spread and commercial use of hybrid MS instruments that combine the accuracy of HRMS with the specificity and sensitivity of MS^n [18]. Accordingly, these hybrid MS

instruments currently bring the best to the world of MS DNA adductomics, although additional work is required to further optimize the use of MS for DNA adductome mapping.

The number of published DNA adductomics methods that make use of hybrid HRMS/MS is very limited. In fact, Balbo et al. were the first – and up until now, only ones - to implement hybrid HRMS/MS technology for DNA adductomics purposes [15]. However, in light of this PhD, a new HRMS-based DNA adducomics methodology was developed and optimized. The potential application of the newly developed method is twofold; i.e. the presence and levels of four DNA adduct types, i.e. O^6-MeG, O^6-CMG, M_1G and CroG, can be assessed by means of a targeted HRMS/MS approach, whilst screening of unknowns is enabled *via* an untargeted full scan HRMS approach. The described analytical method was validated successfully through assessment of specificity, selectivity, linearity, precision and accuracy. In addition, the *in vitro* and *in vivo* application of the newly developed methodology demonstrated highly promising results and future prospects with regard to the investigation of DNA adduct formation following dietary exposure to genotoxic chemicals in the meat-cancer relationship. Furthermore, the developed methodology can also be implemented for other environmental carcinogenesis related research topics.

In parallel, to facilitate data interpretation and compound identification, an in-house diet-related DNA adduct database was established. At the time, and to the best of my knowledge, there is no publically available DNA adduct database. Furthermore, it appears that very few DNA adduct compounds are included in existing chemical databases like e.g. ChemSpider and the Human Metabolome Database. In addition, despite the inclusion of certain DNA adduct compounds in the above-mentioned databases, the provided information is mostly limited (e.g. no fragmentation spectra). The self-constructed in-house DNA adduct database currently contains over 180 diet-related DNA adduct types, mostly including alkylation and oxidation induced DNA adducts. Moreover, based on existing and newly published literature, the database is updated continuously ensuring up-to-date and state-of-the-art research.

1.2 Assessment of N-nitroso compound and lipid peroxidation product formation, metabolic activity and cytotoxicity

Within the previously mentioned DNA adduct database, DNA adduct types formed due to alkylation, oxidation or attack of DNA by LPOs are included. As such, in this work, the formation of genotoxic compounds with alkylating or oxidising properties could be assessed in an indirect manner. Direct assessment of the formation of (alkylating) NOCs or LPOs was only performed on occasion, and the same applies for the determination of metabolic activity and cytotoxicity.

In chapter II, the metabolic activity and cytotoxicity of red *vs.* white meat digests was assessed, specifically focussing on the digestion of chicken, pork and beef. Metabolic activity was determined by measuring short chain fatty acid, indol, phenol, *p*-cresol and ammonia production, whilst cytotoxic effects were monitored by evaluating changes in Caco-2 cell viability. Both cytotoxicity and metabolic activity did not significantly differ between red and white meat digestion. In the same experimental setup, ATNC analysis was performed on stomach, small and large bowel meat digests. The results demonstrated that no ATNC levels could be detected in small and large bowel digests, despite the formation of the NOC-related O^6-CMG DNA adduct during colonic meat digestion. In contrast, NOC-derived NO could be detected in stomach samples, with a trend towards higher ATNC levels upon the digestion of beef. However, due to technical malfunctions during analysis, the validity of these results can be questioned. Even more so since others have previously documented the presence of ATNCs in ileal and fecal samples, moreover reporting a significant increase in ATNC levels following red meat digestion [19, 20]. However, if the non-detection of ATNCs in small and large bowel digests cannot be attributed to technical malfunctions, we must consider the possibility that O^6-CMG formation does not necessarily originate from the production of NOCs during (red) meat digestion. If so, the formation of O^6-CMG (and other potential alkylation induced DNA adducts) could originate from the gastrointestinal production of other DNA alkylating metabolites that are yet to be identified.

MDA is a naturally occurring product of lipid peroxidation with mutagenic and carcinogenic properties [21]; MDA can induce DNA cross-links, frameshift mutations, and base pair substitutions [22, 23]. During several experiments set up over the course of this PhD, the *in vitro* gastrointestinal formation of MDA was monitored on multiple occasions (as described in

chapters II, IV and V). The obtained results were highly consistent; i.e. MDA formation was significantly higher upon red meat (i.e. beef) digestion in comparison to white meat (i.e. chicken) digestion. In addition, the supplementation of myoglobin to beef digestions resulted in a dose-response increase of MDA. We were also able to demonstrate that the addition of calcium carbonate to meat preparations resulted in a decreased formation of MDA (as described in chapter IV). The obtained results align with the heme hypothesis; i.e. heme iron in red meat stimulates the formation of reactive oxygen species and LPOs, including MDA, through the Fenton reaction. Calcium on the other hand, is hypothesized to capture and precipitate heme iron [24], and as such limit the heme iron induced formation of ROS and LPOs. Of course, this is of particular interest since the consumption of calcium-rich dairy products is inversely correlated to CRC risk [25].

In summary, we were able to document that:

1. Red meat digestion results in a significant increase in lipid peroxidation under the form of MDA formation.
2. Addition of myoglobin to meat preparations increases lipid peroxidation under the form of MDA formation.
3. Addition of calcium to meat preparations reduces lipid peroxidation under the form of MDA formation.
4. The earlier reported increased formation of NOCs due to red meat digestion (in comparison to white meat digestion) could not be confirmed.
5. Red meat digestion does not significantly alter (Caco-2) cell viability or metabolic activity (under the form of short chain fatty acid, indol phenol, *p*-cresol and ammonia production).

1.3 Person-dependent patterns in the *in vitro* digestion DNA adductome

With regard to the *in vitro* simulation of digestion, the cultivation of the fecal microbiome of human donors to mimic colonic digestion was indispensable. Moreover, it soon became clear that the composition and metabolic activity of the gut microbiome plays a very dominant role in the colonic digestion and concomitant DNA adduct formation. For example, as was described in

chapter II, colonic metabolic activity (i.e. the formation of SCFAs indol phenol, *p*-cresol and ammonia production) was very much dependent on the fecal donor, as was O^6-CMG formation. Later on, mapping of the *in vitro* DNA adductome also reflected this interindividual variability; i.e. there were significant differences when comparing 'individual' DNA adductomes.

The decribed interindividual DNA adductomic differences are the result of differences in metabolic activity, which stems from the fact that each individual's microbiome is in fact unique [26, 27]. The composition of the microbiome is mostly shaped in early life, but nevertheless modified due to environmental factors like diet, lifestyle social interactions, etc. throughout life [26, 27]. The gut microbiome exerts various physiological functions that are mutually beneficial to the host and microbiome. The gut microbiome is, for example, responsible for the metabolic conversion and uptake of dietary components, competition with pathogens, and regulation of host immune function [28]. However, since the composition and activities of the gut microbiome seem to directly influence cancer (and other chronic disease) susceptibility, it has also become clear that the gut microbiome can just as well exert adverse health effects [28]. More specifically, the gut microbiome can produce detrimental metabolites as a result of mere physiological food metabolisation or actively contribute to the transformation and activation of environmental pollutants [29].

The observed interindividual variations in overall metabolic activity and the DNA adduct forming potential of the gut microbiome may play an important part in interindividual differences in cancer susceptibility. A specific example concerning possible bacterial involvement in DNA adduct formation, that is relevant to the heme hypothesis, concerns the interindividual difference in bacterial N-nitrosation and the hence induced formation of NOCs. Several (facultative) anaerobic bacteria can induce NOC formation *via* nitroreductase and nitrate reductase, and as such lead to NOC-induced DNA adduct formation [28, 30, 31]. In agreement, it has already been demonstrated that nitrate reductase is upregulated in obese individuals as well as inflammatory bowel disease patients [32], which are indeed more susceptible to CRC [25]. Therefore, assessment of nitroreductase and nitrate reductase activity in relation to NOC as well as alkylation induced DNA adduct formation could provide useful insights into individual CRC risk and etiology. This was not assessed in light of this PhD, but should be investigated in follow-up research. Whether or not heme iron can directly or indirectly interfere with bacterial N-nitrosation is of specific interest as well. Since diet shapes the gut microbiome, dietary heme

iron may be a contributing factor and/or directly promote the person-dependent rate of nitrate reductase induced NOC formation.

By analogy, individual gut microbiome variability may also contribute to the observed interindividual differences in DNA adduct formation due to the attack of DNA by ROS and/or LPOs. Oxidation and ROS production are inherent to both mammalian and bacterial energy metabolism. Hence, differences in the composition of the gut microbiota can contribute to shifts in ROS and LPO formation, as well as the gastrointestinal DNA adductome [31, 33].

To conclude, it can be stated that it is very likely that the individual gut microbiome actively drives the person-dependent formation of several (diet-related) DNA adducts. Therefore, future work in line with this PhD project should provide a detailed account of the composition and activity of the gut microbiome (by e.g. metagenomics and metatranscriptomics, respectively) in relation to red and processed meat intake, and the gastrointestinal or fecal DNA adductome. In addition, when conducting *in vitro* experiments using human fecal samples or human *in vivo* experiments, dietary patterns, habits, and other relevant lifestyle factors of the human volunteers should be controlled and/or monitored since this could provide relevant information with regard to interindividual variability.

1.4 Diet-induced shifts in the *in vitro* and *in vivo* DNA adductome

The DNA adductome associated with the digestion of beef significantly differed from that of chicken *in vitro* as well as *in vivo*. Due to the causal link between red meat, but not white meat, consumption and CRC, only DNA adduct types that appeared to be significantly higher in beef *vs.* chicken digests were indeed of specific interest. This can be explained by the fact that not all DNA adduct types are mutagenic and/or carcinogenic, and certain DNA adduct types are readily repaired by DNA repair mechanisms [34]. Hence, DNA adduct formation does not necessarily contribute to cancer risk. Whether or not this is the case for red meat induced DNA adducts is the point at issue.

Two independent *in vitro* and one *in vivo* experiment were set up to assess red meat induced DNA adduct formation. A significant shift in the DNA adductome due to red meat digestion (in comparison to white meat) could be observed throughout. As a result, the increased formation of several putatively detected DNA adduct types could be linked to red meat digestion. In

addition, it was also demonstrated that meat calcium and fat content can induce significant shifts in the DNA adductome, documenting their potential role as modulating factors. As a result, it was confirmed that diet in general, and red meat in particular, can induce DNA damage in the form of DNA adduct formation.

The interindividual variability in the DNA adduct forming potential of the gut microbiome however somewhat impeded straightforward interpretation of DNA adduct data and discovery of DNA adduct biomarkers associated with red meat digestion. Nevertheless, several DNA adduct types could be singled out as they were detected on multiple occasions; i.e. independently throughout both *in vitro* and *in vivo* experimental setups. 7 DNA adduct types could repeatedly and consistently be associated with red meat digestion. These specific DNA adducts of interest are listed in table 1, and discussed briefly below.

O^6-CMG, methyl-G and dimethyl-T (or ethyl-T) are formed due to DNA alkylation, which may occur due to endogenous as well as exogenous exposure to alkylating chemicals like NOCs [34]. *In vivo* as well as *in vitro* methylation and ethylation of G and T has previously been documented, but only O^6-CMG was previously linked to red meat consumption [34, 35]. Malondialdehyde-2x-G (M_2G) and Malondialdehyde-3x-C (M_3C) are DNA adduct types that originate from the interaction between MDA and DNA [21, 36]. The earlier discussed finding that beef digestion promotes MDA formation, supports these results. In addition, the formation of heptenal-G also corresponds to the documented increase of lipid peroxidation since heptenal is another well-known LPO [37]. Carbamoylhydroxyethyl-G has previously been detected *in vivo* in association with acrylamide exposure [38]. Nevertheless, nothing about the carbamoylhydroxyethyl-G structure suggests that the formation of this DNA adduct type is strictly limited to the attack of the G nucleobase by acrylamide; i.e. other diet-related genotoxins, including NOCs and/or LPOs, may be able to contribute to its formation.

The retrieval of these alkylation and/or oxidation induced DNA adduct types in relation to red meat digestion, is in support of the heme, NOC and lipid peroxidation hypotheses. It furthermore suggests that DNA adduct formation may contribute to red meat consumption related CRC risk, which should definitely be further investigated.

Table 1. DNA adduct markers that were assigned to red meat digestion in this work.

DNA adduct name	Documented in chapter nr.	Context	Test	p-value or VIP score
O^6-Carboxymethyl-G	II	*In vitro* digestion of beef (compared to chicken)	ANOVA	$p = 0.05$
	IV	*In vitro* digestion of beef (compared to chicken)	Student's t-test	$p < 0.01$
	IV	Increased *in vitro* formation due to addition of calcium	Student's t-test	$p = 0.04$
	V	*In vitro* digestion of beef (compared to chicken)	Student's t-test	$p = 0.05$
Dimethyl-T or ethyl-T	IV	*In vitro* digestion of beef (compared to chicken)	Sieve™ pairwise comparison	$p = 0.02$
	V	*In vitro* digestion of beef (compared to chicken)	Simca™ analysis	VIP = 1.95
Methyl-G	V	*In vitro* digestion of beef (compared to chicken)	Simca™ analysis	VIP = 1.23
	VI	Increased *in vivo* formation due to addition of fat	Student's t-test	$p = 0.03$
Malondialdehyde-2x-G	IV	*In vitro* digestion of beef (compared to chicken)	Sieve™ pairwise comparison	$p = 0.05$
	VI	*In vivo* digestion of beef (compared to chicken)	GENE-E marker selection	$p = 0.02$
Heptenal-G	V	*In vitro* digestion of beef (compared to chicken)	Student's t-test	$p = 0.05$
	VI	*In vivo* digestion of beef (compared to chicken)	Student's t-test	$p = 0.03$

Table 1 continued.

DNA adduct name	Documented in chapter nr.	Context	Test	p-value or VIP score
Carbamoylhydroxyethyl-G	V	*In vitro* digestion of beef (compared to chicken)	Student's t-test	$p = 0.03$
	VI	*In vivo* digestion of beef (compared to chicken)	Student's t-test	$p = 0.04$
Malondialdehyde-3x-C	IV	*In vitro* digestion of beef (compared to chicken)	Sieve™ pairwise comparison	$p < 0.01$
	V	*In vitro* digestion of beef (compared to chicken)	Student's t-test	$p = 0.01$

1.5 Critical Notes

1.5.1 *In vitro* model *vs. in vivo* situation

In Chapter II, IV and V, red *vs.* white meat digestion experiments were performed by means of a static *in vitro* digestion model, sequentially exposing the meat to simulated mouth, gastric, small and large intestinal digestion. Said *in vitro* model is very versatile and as such ideally suited for mechanistic explorative work. Furthermore, the use of an *in vitro* model is preferred over the use of an *in vivo* (e.g. rodent) model due to ethical considerations, whilst the first is also less costly and time-consuming than the latter. In contrast, the employed *in vitro* model demonstrates certain flaws. More specifically, the model does not allow absorption of digestive metabolites and/or interaction with the intestinal wall. Therefore, genotoxic metabolites formed during simulated digestion of meat, could not directly interact with the intestinal mucosa (and its DNA). In chapter II, Caco-2 DNA was added to the meat digests to measure the interaction between genotoxic meat digestion metabolites and human DNA. However, in contrast with initial expectations, O^6-CMG levels did not differ with and without addition of Caco-2 DNA. Therefore, it was assumed that O^6-CMG levels in meat digests originated from the direct interaction with bacterial DNA, which was overly abundant in the digestion flasks. By analogy, DNA adducts could also have originated from the interaction with DNA contained in the digested meat, although the exact origin of the DNA adduct containing DNA was not investigated at the time. As a result, throughout chapter II, IV and V, prokaryote DNA adduct formation was used as a proxy for eukaryote DNA adduct formation. Although prokaryote and eukaryote DNA demonstrate some distinct differences, the DNA building blocks are chemically identical. In addition, studies on DNA damage and DNA repair often use prokaryotic DNA as a tool to investigate similar processes in eukaryotic DNA [39]. Hence, it was assumed that the interaction between genotoxic molecules and the nucleobases in eukaryotic and prokaryotic DNA are similar, permitting the use of prokaryotic DNA adduct formation as a model for eukaryotic DNA adduct formation in the employed *in vitro* digestion model. Moreover, to the best of my knowledge, there currently is no valid *in vitro* model which allows the direct interaction with human DNA to remedy this issue.

Previously in this chapter, 7 potential red meat digestion markers were singled out, taking into account the *in vivo* rat data as well as the *in vitro* GI digestion data obtained in light of this thesis. Unfortunately, the obtained *in vitro* and *in vivo* DNA adductomic data cannot be compared and

thus support each other directly since the matrices under investigation differ (liver and gut tissue *vs.* gut luminal content). A direct comparison between *in vitro* and *in vivo* GI DNA adduct formation would have been possible if the DNA adductome of the luminal content of the rat guts was collected and investigated. Unfortunately, this was not feasible due to 2 different reasons: (1) the small amount of gut luminal contents and thus also extractable DNA, and (2) the need for a separation of eukaryotic from prokaryotic DNA in order to properly validate the *in vitro* results; i.e. the *in vitro* model allows prokaryotic DNA adduct formation (as was discussed previously), whilst the rat model reflects prokaryotic as well as eukaryotic (due to shedding of epithelial cells) DNA adduct formation. Isolation of eukaryotic cells from the rest of the intestinal content has previously been accomplished in humans [35], but would require further optimisation for implementation in rats.

1.5.2 DNA adduct formation *vs.* epigenetics

The field of epigenetics studies the occurrence of inheritable chemical modifications in gene expression that cannot be explained by alterations in the underlying DNA sequence. More specifically, it has become clear that environmental influences can alter DNA packaging and expression, play a role in disease susceptibility, and can furthermore be passed on to subsequent generations [40]. One of the most extensively studied types of epigenetic changes is DNA methylation, which is involved in gene transcription. In eukaryotes, this involves methylation at the C5 position of cytosine, leading to the formation of 5-methylcytosine (5-MeC) [41]. In prokaryotes however, it is N^6-methyladenine (N^6-MeA) that can act as a (post-replicative) epigenetic signal [42]. As has already become clear throughout this thesis, the methylation of DNA nucleobases can not only occur naturally in light of gene expression regulation, but can also be induced through environmental exposure to DNA-alkylating chemicals. Taking this into account, there is an important overlap between chemically induced DNA adduct formation and epigenetics. In case of 5-MeC in eukaryotes or N^6-MeA in prokaryotes, one may wonder whether certain environmental exposures induce this type of DNA methylation *via* direct attack of the DNA nucleobases, or whether the exposed organism actually responds to the environmental exposure by altering its gene regulation. There is no way to easily distinct one from the other.

1.5.3 DNA adduct profiling *vs.* fingerprinting

When applied in full scan acquisition, the UHPLC-HRMS DNA adductomic method (as described in Chapter III) allows untargeted mapping or fingerprinting of all positive and negative ions in each extract. Preprocessing of the hence obtained data by means of Sieve™ software enables transformation of ion intensities into a matrix of features (m/z - RT pairs) using peak detection, alignment and area extraction algorithms, followed by normalization, scaling and transformation to correct for systematic and technical variability. Subsequently, differences between (sub)groups of interest (i.e. red *vs.* white meat), and potential marker molecules can be discovered by means of multivariate data mining (i.e. Simca™ software). Up until this point, analysis and data processing is blind, meaning that accurate mass is not linked to a possible identity. However, from this point on, the accurate masses of ions of interest (i.e. possible markers) are matched with the in-house DNA adduct database, and in this work only 'matched' ions were reported. This can provide a certain degree of bias since (1) only known types of DNA adducts are included in the DNA adduct database, and (2) this work mainly focused on the investigation of alkylation and/or oxidation induced DNA adduct types. As a result thereof, ions that may very well correspond to unknown adducts were not reported, although some of these ions did display marker potential (e.g. VIP score > 1).

1.5.4 DNA adduct formation due to the digestion of chicken

Throughout this thesis, exploration of the obtained data and discussion of the results was hypothesis-driven, meaning that there was a specific focus on red meat digestion related DNA adduct formation, but not white meat digestion related DNA adduct formation. DNA adduct types that decreased upon red meat digestion and/or increased upon white meat digestion were reported in heat maps and tables, but not discussed because of (1) the vast amount of data and work that was generated, and (2) the fact that the (excessive) digestion of white meat has not been linked to CRC risk. Therefore, increased formation of certain types of DNA adducts due to the digestion of chicken, as a model for white meat, was not deemed relevant. Nevertheless, there was no distinct quantitative difference in the total number of retrieved DNA adduct types upon digestion of chicken or beef, and therefore, it should be noted that the digestion of white meat can also affect the DNA adductome, and that beef, as a model for red meat, does not

induce an overall increase in DNA adduct formation compared to white meat. The same rationale applies for low *vs.* high fat content, and whether or not $CaCO_3$ or myoglobin is added.

1.5.5 Statistical interpretation of the data: suggestions for improvement

DNA adductomics is a relatively new omics branch and therefore, a mostly uncharted field of research. As a result, the approach for untargeted data (pre)processing and interpretation requires further exploration and optimisation. In light of this PhD, several types and means of data processing and interpretation were explored; i.e. ToxFinder™ profiling, Sieve™ pairwise comparison and Simca™ modelling. As such, univariate as well as multivariate statistics were employed to search for relevant trends in the obtained data. However, due to the vast amount of data that was generated, extensive statistical validation of the obtained results was not always feasible, possibly leading to over-interpretation of the obtained results.

With regard to univariate procedures, ANOVA should be preferred over 'simple' t-testing, allowing a more profound evaluation of the role of fixed and random variables, as well as interactions. Furthermore, when conducting multiple testing, false discovery rate controlling procedures (e.g. Benjamini-Hochberg) should be employed to correct for type I errors. With regard to multivariate testing, based on for example OPLS-DA, extensive validation of the obtained model (and the hence retrieved information) is required, employing a training and test dataset to evaluate model prediction performance and avoid over-fitting.

Concerning the interpretation of all *in vitro* data, it should be kept in mind that digestions performed with the same fecal inoculum (e.g. comparing red *vs.* white meat digestion using the fecal inoculum of a certain individual) or different fecal inocula obtained from the same person are not independent. Therefore, the hence obtained results should be assessed using dependent statistical tests (e.g. repeated measures ANOVA or repeated measures regression analysis).

Lastly, it should be noted that the true value and meaning of a red meat digestion marker can only be assessed in a sufficiently large cohort. Nevertheless, explorative as well as mechanistic experiments, like the work conducted over the course of this PhD, are indispensable to gain a more profound knowledge of underlying causes and pathways.

2. FOLLOW-UP RESEARCH

2.1 Human case-control studies

DNA adductomics is particularly well-suited for research on the exposure of the human body to both known and unknown endo- and exogenous hazardous chemicals and any subsequently formed DNA adducts [16]. Nevertheless, the search for answers does not end with DNA adduct mapping or biomarker establishment, as the described top-down approach does not evidently link genotoxin exposure to a certain environmental factor as a causal risk factor on the one hand, or disease outcome on the other [4]. Any information obtained from top-down omics studies will only prove its value if combined with bottom-up targeted analyses in both long-term studies and purposeful short-term intervention studies [1, 4]. Therefore, to confidently link cause and effect, future DNA adductomics studies aiming to investigate CRC initiation, promotion and progression should be based on human case-control cohorts. More specifically, we are in need of observational as well as interventional case-control studies to firstly, confidently link dietary (and other lifestyle) habits to DNA adductomic shifts, and secondly, link these DNA adductomic changes to actual CRC risk and development. To this purpose, the DNA adductome should be monitored in tumoral tissue, blood and feces of CRC patients and healthy volunteers, after which the obtained DNA adductomic data can be linked to dietary habits (i.e. assessed by means of a food frequency questionnaire), focusing on red and processed meat consumption, and disease status (e.g. healthy *vs.* adenoma *vs.* carcinoma).

2.2 Obstacles concerning (*in vivo*) DNA adductomics

Insufficient knowledge of DNA adduct fragmentation patterns and limited availability of DNA adduct standards currently act as a bottleneck for the full characterization and correct identification of untargeted and unknown DNA adducts with MS [16]. In this context, the need for accurate mass measurements is indisputable, whereas the establishment of a database to assemble all information on chemical structure and characteristics of DNA adducts, fragmentation patterns, stability, prevalence and origin (= initiating genotoxin + route of exposure) would provide a major advance by facilitating investigation of DNA adduct formation and its potential role in different pathophysiological pathways. In addition, technological as well as methodological improvements may provide the means to tackle these issues (discussed further on).

The relatively low concentration of DNA adducts in biological matrices forms an additional obstacle. As was mentioned previously, DNA adducts are not abundantly present in DNA. Furthermore, the concentration of DNA in several biological matrices is relatively low as well. In contrast, a considerable amount of purified DNA (> 50 µg) is required for DNA adduct analysis purposes. Hence, sample size requirements limit DNA adduct research possibilities. As was previously explained in chapter I, this issue could possibly be tackled by the collection and analysis of appropriate surrogate tissue(s). However, this can only be implemented if the DNA adductome (or a specific fraction of interest) of the surrogate tissue directly and correctly reflects that of the target tissue. However, at the time, and to the best of my knowledge, such correlations have not sufficiently been investigated. Hence, until additional research concerning the DNA adductome of surrogate *vs.* target tissue has been performed, the obstacle of required sample size remains.

3. POTENTIAL TECHNOLOGICAL AND METHODOLOGICAL IMPROVEMENTS IN THE FIELD OF DNA ADDUCTOMICS

3.1 Mass Spectrometric tools that are currently in use

3.1.1 Triple Quadrupole Tandem Mass Spectrometry

LC-ESI-tandem MS (LC-MS/MS) by means of triple quadrupoles is currently the most applied technique/instrument for the targeted quantification of DNA adducts [17]. With LC-MS/MS, mapping of the DNA adductome is enabled through monitoring of the constant neutral loss (CNL) of 2'-deoxyribose (116 Da) from positively ionized 2'-deoxynucleoside adducts. This approach is demonstrated for four different adducts in figure 2, where the difference between the precursor ion and the base peak is always 116 Da [6]. One can focus on all $[M+H]^+$ to $[M+H - 116]^+$ transitions by applying a full scan approach in Q3, the third quadrupole, or alternatively use selected reaction monitoring (SRM) to view $[M+H]^+$ to $[M+H - 116]^+$ transitions in a more narrow, selected range. The narrow range of SRM can be compensated by multiple injections of the same sample, which are then analyzed in different mass ranges, although this requires more time for analysis [16].

The group of Kanaly *et al.* was one of the first to develop and apply an LC-MS/MS method for mapping of the DNA adductome. Analysis of human lung and esophagus DNA samples revealed the possible presence of more than 1000 putative DNA adducts in each tissue type. The use of analytical standards and isotope dilution allowed full identification of seven DNA adducts [10, 43]. This demonstrates the vast amount of data that can be generated and the amount of time involved in positively identifying the adducts within a particular sample; the analysis itself takes 28 to 60 min. per sample and the authors describe that the time required for data processing and confident identification is 'manageable'. Examples of similar LC-MS/MS applications include work by several research groups, demonstrating the popularity of this type of instrumentation for DNA adductomics [17, 44-51].

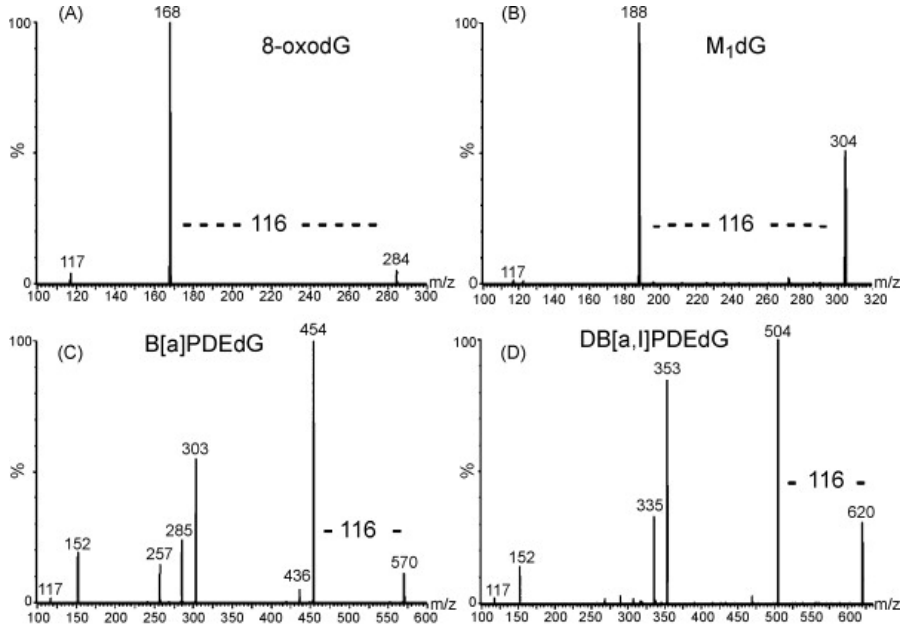

Figure 2. ESI MS/MS mass spectra documenting $[M+H]^+$ to $[M+H-116]^+$ transitions of four DNA different adducts; (A) 8-oxo-dG, the main oxidative stress related DNA adduct; (B) M1dG, the main MDA adduct; (C) B[a]PDEdG, the main benzo[a]pyrene diol epoxide DNA adduct; and (D) DiB[a]PDEdG, a dibenzo[a,1]pyrene diol epoxide DNA adduct. Adapted from Farmer & Singh P.B.; Singh, R. Mutat. Res.-Rev. Mutat. 2008, 659, 68-76 [6], copyright 2008 Elsevier.

An alternative approach to monitoring of the mutual loss of 2-deoxyribose, is the detection of altered DNA nucleobases instead of altered nucleosides. Inagaki *et al.* reported the presence of

characteristic fragment ions for guanine at m/z 152 (≈ protonated guanine) and 135, which corresponds to fragmentation of the NH_2 group, and for adenine at m/z 136 (≈ protonated adenine) and 119 [9], which is presumably the corresponding fragmentation although the authors did not show the data. Confirming part of these findings, Gregson *et al.* also documented deamination upon collision induced fragmentation of protonated guanine [52], whilst other independent research groups have also reported the occurrence of a product ion with m/z 152 for guanine [53, 54] and m/z 136 for adenine [55, 56] using different systems and focusing on different DNA adduct types. However, at the present time, it is not clear whether those exact same ions are formed upon fragmentation of all purine DNA adduct types.

The aforementioned research demonstrates that tandem MS can reveal hundreds of putative DNA adducts in DNA samples and thus holds great potential for biomarker discovery. However, one disadvantage of tandem MS/MS is the loss of sensitivity with CNL or the need for SRM transition optimization for each different DNA adduct in order to achieve sufficient detection sensitivity with pseudo-CNL [16, 17]. Secondly, since triple quadrupoles only allow low resolution data acquisition, and DNA adduct databases providing MS/MS spectra are not available, DNA adduct identity confirmation is dependent on the availability of analyte standards or the use of additional analytical techniques. This renders triple quadrupole mass measurements to be less suited for untargeted compound analysis and confident compound identification compared to HRMS, e.g. TOF and orbitrap, which is discussed further on [16].

3.1.2 Ion Trap Mass Spectrometry

Ion trap MS-analyzers allow multistage scan events (MS^n) that provide additional structural information. Just like most triple quadrupole methods, ion trap DNA adduct analysis depends on the detection of the neutral loss of the 2'-deoxyribose group. Bessette, Turesky and co-workers describe the use of a linear ion trap for data-dependent LC-MS^3 (DD-CNL-MS^3), where first, the detection of a DNA adduct ion (listed in a targeted mass-list) in a limited m/z scan range leads to MS^2 acquisition. Subsequently, the detection of the $[M+H - 116]^+$ ion amongst the top ten of the most abundant MS^2 ions triggers MS^3 fragmentation. Bessette *et al.* used this acquisition type to study the formation of tobacco-associated DNA adducts of certain aromatic amines, HCAs, PAHs and aldehydes in rat livers, human hepatocytes and buccal cells [11]. MS^3 acquisition or multistage MS^n scanning in general, seems a major advancement compared to MS^2

CNL scanning techniques since MS^n provides a higher specificity and further DNA adduct characterization [11, 16]. Unmistakable identification with the ion trap occurs through evaluation of the MS^n product ion spectrum and co-elution with an analytical standard. If necessary (e.g. no analytical standard available), the use of additional analytical techniques using accurate mass measurements may assist in the identification of unknowns. Co-workers of Bessette and Turesky applied the DD-CNL-MS^3 approach in research on 4-aminobiphenyl, HCA and aristolochic acid-related aristolactam DNA adducts with a clear focus on targeted DNA adduct detection [12, 57]. Pietsch *et al.* adapted the method described by Bessette *et al.* to study DNA adduct formation by Illudin S, an antitumoral agent. They were able to study known DNA adducts in a colon cancer cell line, but were unable to detect or identify any untargeted DNA adducts [58]. This suggests that although the ion trap and DD-CNL-MS^3 method have proven their worth for structural characterization, identification and quantitation of (a limited number of) targeted DNA adducts, the low resolution methodology appears less suited for holistic, untargeted omics applications, including DNA adductomics [59]. Figure 3 demonstrates compound identification by means of MS^3 fragmentation patterns for three different DNA adducts of the HCA MeIQx (deoxyguanosine-N^2-MeIQx, deoxyguanosine-C^8-MeIQx, and deoxyadenosine-C^8-MeIQx) [11] whereby the MS^3 fragmentation patterns obtained allow confident compound identification.

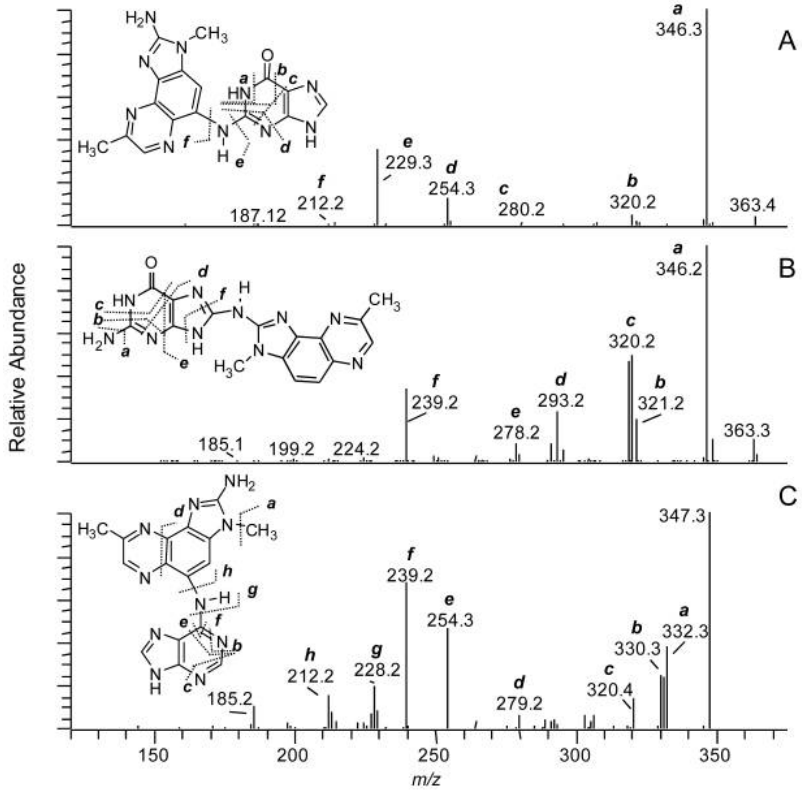

Figure 3. CNL-MS^3 product ion spectra of three MeIQx DNA adducts, from HCA exposure, clearly showing multiple fragmentations within the adducts; (A) dG-N^2-MeIQx, (B) dG-C^8-MeIQx, and (C) dA-N^6-MeIQx. Reproduced from Bessette, E. E.; Goodenough, A. K.; Langouet, S.; Yasa, I.; Kozekov, I. D.; Spivack, S. D.; Turesky, R. J. Anal. Chem. 2009, 81, 809-819 [11], copyright 2009 American Chemical Society.

3.1.3 Time of Flight High Resolution Mass Spectrometry

TOF instruments are most commonly used for qualitative analysis as a stand-alone instrument, although coupling to a second mass spectrometer offers several opportunities for DNA adductomics studies. Recently, Giese and co-workers developed a MALDI-TOF/TOF method (MALDI = matrix-assisted laser desorption/ionization) to enable untargeted DNA adduct detection (preceded by HPLC separation), facilitating investigation of unknown DNA adducts. To this purpose, they employed a tedious but highly profitable sample preparation procedure based on benzoylhistamine labeling of altered nucleotides. This approach enabled the specific detection of altered deoxynucleotides with increased sensitivity and specificity (noise was reduced due to the use of negative ionization) in a semi-quantitative manner [13, 60].

By coupling of a TOF-MS to a quadrupole, the resulting hybrid instrument can also be easily employed for both identification and quantitation purposes since accurate mass measurements are important for confident compound identification and can also eliminate spectral noise due to matrix interferences [18]. Esmans, Van den Driessche and colleagues published research [14, 61] on the use of a QTOF instrument for DNA adduct screening and characterization. Unfortunately, and to the best of our knowledge, both the use of the QTOF (quadrupole coupled to TOF) and MALDI-TOF/TOF instruments for DNA adductome mapping has not been explored further which may be due to the lack of available instrumentation in appropriate research laboratories as tandem MS instrumentation has been favoured in recent years.

3.1.4 Orbitrap High Resolution Mass Spectrometry

Orbitrap technology enables very accurate mass detection due to a high resolving power and mass accuracy. The technology is particularly suited and implemented for small molecule analysis and untargeted omics applications. The orbitrap is often coupled to an ion trap instrument (early on) or a quadrupole (later on), but can also be used as a standalone instrument [18].

Recently, Balbo *et al.* developed a high resolution DD-CNL-MS^3 method for DNA adductomics purposes, using a linear ion trap-orbitrap system [15]. Within the described application, the orbitrap ensures accurate mass measurements resulting in determination of possible elemental composition, selective identification of DNA adducts and avoidance of false positives. The CNL $[M+H]^+$ to $[M+H-116]^+$ transition triggers MS^3 acquisition, further contributing to molecular structure data and assisting with identification of untargeted DNA adducts (demonstrated in figure 4). Therefore, this methodology appears to be suitable for wider application to adductomics areas of research.

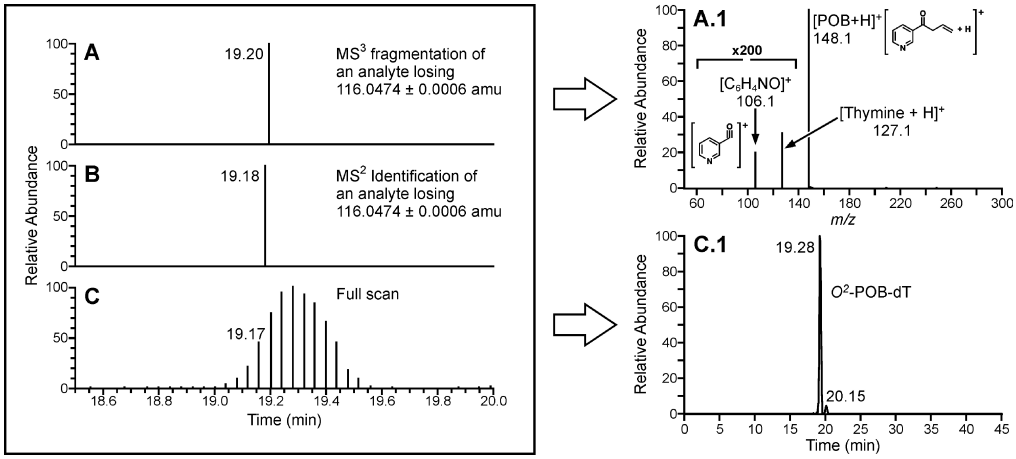

Figure 4. Demonstration of the CNL-MS^3 high-resolution/accurate mass adductomic approach with an O^2-POB-dT standard (a tobacco-specific nitrosamine related DNA adduct). (A) MS^3 scan event triggered by a mass difference of 116.0474 amu between an ion mass in the full scan (C) and an ion mass in the corresponding MS^2 spectrum (B). (A.1) MS^3 spectrum of O^2-POB-dT, (C.1) accurate mass full scan ion chromatogram of O^2-POB-dT (*m/z* = 390.1660 amu). Reproduced from Balbo, S.; Hecht, S. S.; Upadhyaya, P.; Villalta, P. W. Anal. Chem. 2014, 86, 1744-1752 [15], copyright 2014 American Chemical Society.

3.2 Perspectives for Mass Spectrometry-based DNA adductome mapping

3.2.1 Hybrid High Resolution Mass Spectrometry

For years, triple quads have dominated the field of trace quantitation [18]. Yet, when conducting DNA adductome mapping, tandem MS encounters some difficulties regarding DNA adduct identification; since accurate mass data are not available and, at the time, sufficient knowledge on MS/MS spectra of DNA adducts is somewhat lacking. This necessitates that comparison with analytical standards and/or the use of other analytical techniques are essential for confident compound identification. High resolution MS provides accurate mass measurements and thus more information regarding compound mass, elemental composition and identity. New hybrid systems like QTOF and ion trap-orbitrap may not always surpass triple quadrupole instruments for low level quantitation of DNA adducts in terms of peak areas, but full scan HRMS acquisition always results in lower signal to noise ratios compared to low resolution MS due to elimination of noise. Therefore, comparable limits of detection and limits of quantification for high resolution hybrids and triple quads are definitely within reach. In any case, hybrid HRMS

systems offer indisputable advantages through accurate mass detection, which renders them to be an excellent tool for omics applications [18].

Besides the quadrupole-orbitrap instrument I implement(ed) (i.e. the Q-Exactive™), the QTOF [61], and the linear ion trap-orbitrap [15], other hybrid HRMS systems such as the quadrupole ion trap-TOF and linear ion trap-fourier transform cyclotron resonance MS could be highly accommodating for DNA adductome mapping [62, 63]. Of course, alternative applications of some of the currently used hybrid HRMS technologies could also be explored. For example, a quadrupole-orbitrap instrument like the Q-Exactive™ can be operated in different acquisition modes; full scan MS, selected ion monitoring (SIM) MS, MS^2, full scan data dependent MS^2 (DD-MS^2), SIM-DD-MS^2 and neutral loss DD-MS^2 (NL-DD-MS^2), enabling different approaches for targeted and untargeted analysis of complex biological matrices [64]. In particular, the use of NL-DD-MS^2 for untargeted DNA adduct detection should be explored further as this approach can provide $HRMS^2$ spectra of DNA adducts characterized by the loss of e.g. 2'-deoxyribose ($[M+H]^+$ to $[M+H - 116]^+$ transition) upon fragmentation; a distinctive feature of nucleoside DNA adducts that has been commonly exploited for DNA adductomic research by means of both triple quads and ion traps [10, 11]. Employment of the neutral loss of 2'-deoxyribose by nucleosides, and the potentially characteristic loss of protonated bases (as reported by Inagaki and co-workers [9]) during DNA adduct fragmentation, could prove to be very rewarding as it allows analysts to focus their attention on the detection of potential DNA adduct biomarkers exclusively by ignoring all non-DNA adduct originating ions and molecules.

3.2.2 Chromatographic Innovations

As MS is usually coupled to chromatography, further advances could still be achieved by means of modern LC techniques. Within this framework, the use of capillary or nano capillary LC coupled to micro- or nano-ESI-MS could provide a rise in sensitivity [8, 17]. Due to the lower sample flow rates of capillary LC, the ionization and ion sampling efficiency in the electrospray source increase significantly, resulting in a higher amount of ions in the MS system, an improved sensitivity and low mass detection limits [8]. With micro- or nano-ESI, a higher electrospray efficiency and improved MS sensitivity are achieved in the same manner [65]. Both (nano) capillary LC and micro- or nano-ESI-MS have been implemented for targeted DNA adduct analysis [66-68] and allow sensitive DNA adduct analysis with a limited amount of sample.

To eliminate non-altered nucleosides from the sample, two-dimensional (2D-)LC can be implemented [68]. 2D-LC is another on-line chromatography application that could definitely assist with detailed DNA adduct mapping because this technique allows one sample (or its most interesting 'section') to be chromatographically separated twice (with 2 different columns), significantly adding to the separation power required for the analysis of complex biological samples like DNA. 2D-LC has already demonstrated its potential in metabolomics and proteomics [69], and has been used for DNA adductomics at least once by Singh *et al.*, who used a trap column to isolate PAH-dihydrodiolepoxide DNA adducts in order to facilitate subsequent separation by means of an analytical column thereafter [17]. This approach enabled an increased sample throughput and a significant reduction of ionisation suppression and other matrix effects. Besides the elimination of unmodified DNA building blocks by means of a trap column, which significantly enhances the sensitivity of the analysis and also reduces the risk of artifacts [8], 2D-LC also has the potential to assist with the combined and more adequate separation of different types of DNA adducts with different chemical attributes during one single chromatographic run when using two analytical columns. Unfortunately, according to the available literature, this has not been investigated yet.

An additional technique that could be used to achieve an increase in sensitivity, consists of miniaturized separation techniques like LC-chip [17]. This state-of-the-art development improves sensitivity by a gain in ionization efficiency and also significantly reduces the required sample size [70, 71]. However, up to date, there are very few promising papers on DNA adduct analysis by means of LC-chip MS, although the technique was introduced over a decade ago. It appears that the specialized nature of LC-chip technology and the need for specific LC and MS equipment may pose important restrictions for its widespread application. Although the technique definitely seems very promising for DNA adduct biomarker research, its optimisation and subsequent application seems to be rather complex and difficult; e.g. Bani-Yaseen *et al.* documented persistent problems with the separation of similar molecular structures [72]. In contrast, Vouros and co-workers were able to use (commercialized) HPLC-chip MS methodology quite easily for the detection of dG-C8-4-ABP (a 4-aminobiphenyl DNA adduct), although they do not discuss its optimisation and practical use in detail [66].

Unfortunately, none of the above-mentioned techniques have been applied for untargeted DNA adductome mapping, merely leaving us with the promise of a giant leap forward in this field of research. Thus, the potential of these chromatographic innovations has, at least in part, been

demonstrated for targeted DNA adduct analysis, but still needs to be established and confirmed for DNA adductomics purposes.

4. JOINING FORCES TO MAXIMIZE RESEARCH OUTPUT: FUSED OMICS

4.1 Interrelated fields of research

The human metabolism is heavily influenced by e.g. genetics, the gut microbiome, and other external pressures (= the exposome) besides diet [73]. Hence, in the context of diet-related CRC initiation and promotion, in-depth investigation of several of those interfering factors by means of omics applications that are complimentary to DNA adductomics, offers promising perspectives. In light of CRC research, metabolomics as well as metagenomics are of specific interest.

4.1.1 Metabolomics

A metabolic fingerprint can provide the most accurate assessment of the biological state of an individual because it documents all metabolites produced during metabolic processes in that individual's cells, tissues and organs, and is furthermore capable to capture signals from the exposome. Both qualitative and quantitative changes in the metabolome reflect manifesting (patho)physiological changes at a very early stage, e.g. way before changes in the architecture of cancerous cells could be observed [74]. Therefore, assessment of the metabolome provides a very powerful tool for a large variety of cancer (biomarker) studies [73].

The metabolome comprises both polar and non-polar small molecules, which can be mapped by respectively (polar) metabolomics and lipidomics. Both polar metabolomics and lipidomics studies have already demonstrated their relevance and potential by enabling more in-depth investigation of disease pathophysiology and disease biomarker discovery on multiple occasions [75, 76]. More specifically, metabolomics has already allowed investigation of shifts in the gut metabolome upon red meat digestion (in comparison to white meat), revealing several metabolite markers that may be of importance with regard to the development of CRC [77]. In addition, it

has been demonstrated that the polar metabolome as well as the lipidome significantly differ for CRC patients and healthy individuals. Apparently, CRC patients demonstrate a distinctly different metabolic pattern, which contains marker molecules that can be linked to e.g. the tricarboxylic acid cycle, amino acids and fatty acids metabolism [75].

4.1.2 Metagenomics

Metagenomics encompasses the genomic analysis of entire communities of microorganisms, e.g. in the human gut [78]. As it has become clear that the human intestinal microbiota play an important role in several (patho)physiological processes, investigation of gut microbial composition is highly relevant to the field of CRC research. After all, the colonic epithelium is exposed directly to the metabolic products of the gut microbiome. Recently, the dominant interfering role of the gut microbiome in relation to CRC has become indisputable [31, 79] as several studies using state-of-the-art metagenomics have demonstrated that the gut microbiome of CRC patients significantly differs from that of healthy subjects [80] through the presence and/or higher abundance of certain pathogenic (e.g. *E. coli*) as well as commensal (e.g. *Bacteroides* spp.) species [81]. More specifically, it has become clear that the gut microbiome's end products exert DNA damaging effects (e.g. DNA adduct formation) as well as induce cell proliferation, apoptosis and differentiation that can ultimately lead to CRC development [81]. Hence, the gut microbiome, and its metabolic products, strongly influence whether someone develops CRC, or not [31, 81], rendering metagenomics to be a field of interest in light of CRC research.

4.2 The prospect of fused omics

Combining DNA adductomics with metagenomics and metabolomics (including polar metabolomics as well as lipidomics) can provide highly relevant information with regard to CRC initiation, promotion and progression. At the time, data obtained by means of different omics methodologies are processed separately, requiring integration later on. A high-end bio-informatic platform that simultaneously integrates different types of omics data (from a different origin) would however constitute a significant added value and enable more in-depth CRC research. Concomitantly, reports from literature demonstrate that a fused strategy leads to higher accuracy in prediction and more exhaustive description of disease profile; e.g. fusion of proteomics and

metabolomics allowed better discrimination of healthy *vs.* diseased rats in a pre-clinical multiple sclerosis rat model [82, 83]. The fused omics approach also provided insight into the modification of metabolic pathways by the disease [83], rendering fused omics to hold great potential for cancer research in its many aspects as well. Hence, *via* the unprecedented coverage of biological molecules related to disease pathogenesis, the fusion of different omics applications into a single fused omics platform is expected to take cancer research in general, and CRC research specifically, to the next level [84]. As such, a multidisciplinary fused omics approach (presented in figure 5) represents the most efficient and comprehensive way forward.

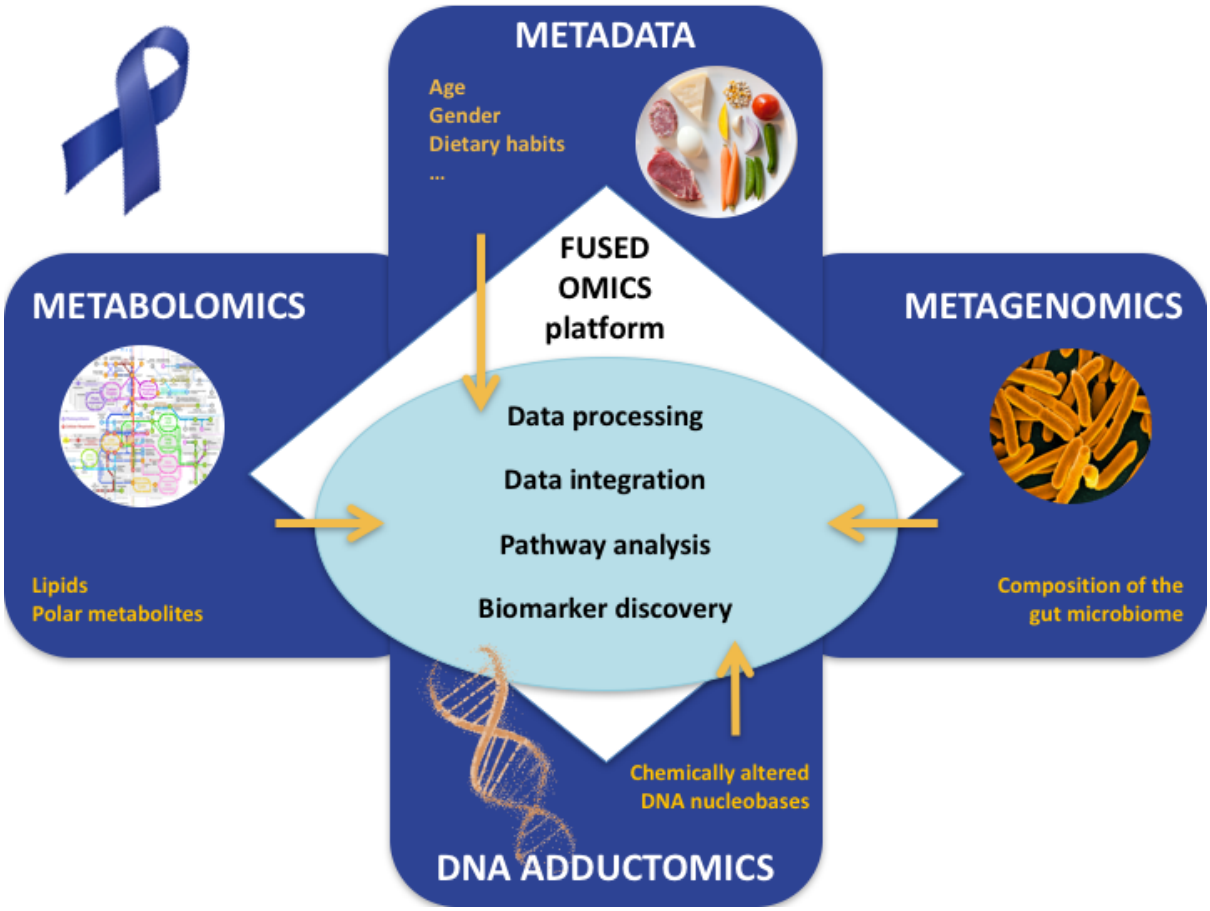

Figure 5. Schematic overview of the envisioned fused omics platform.

REFERENCES

[1] S.S. Coughlin, Toward a road map for global -omics: a primer on -omic technologies, American journal of epidemiology, 180 (2014) 1188-1195.
[2] S.M. Rappaport, Discovering environmental causes of disease, Journal of epidemiology and community health, 66 (2012) 99-102.
[3] P.J. Lioy, S.M. Rappaport, Exposure science and the exposome: an opportunity for coherence in the environmental health sciences, Environmental health perspectives, 119 (2011) A466-467.
[4] C.P. Wild, The exposome: from concept to utility, International Journal of Epidemiology, 41 (2012) 24-32.
[5] M.W. Himmelstein, P.J. Boogaard, J. Cadet, P.B. Farmer, J.H. Kim, E.A. Martin, R. Persaud, D.E. Shuker, Creating context for the use of DNA adduct data in cancer risk assessment: II. Overview of methods of identification and quantitation of DNA damage, Critical reviews in toxicology, 39 (2009) 679-694.
[6] P.B. Farmer, R. Singh, Use of DNA adducts to identify human health risk from exposure to hazardous environmental pollutants: The increasing role of mass spectrometry in assessing biologically effective doses of genotoxic carcinogens, Mutation Research/Reviews in Mutation Research, 659 (2008) 68-76.
[7] P.B. Farmer, K. Brown, E. Tompkins, V.L. Emms, D.J. Jones, R. Singh, D.H. Phillips, DNA adducts: mass spectrometry methods and future prospects, Toxicology and applied pharmacology, 207 (2005) 293-301.
[8] R. Singh, P.B. Farmer, Liquid chromatography-electrospray ionization-mass spectrometry: the future of DNA adduct detection, Carcinogenesis, 27 (2006) 178-196.
[9] S. Inagaki, H. Hirashima, Y. Esaka, T. Higashi, J. Min, T. Toyo'oka, Screening DNA Adducts by LC–ESI–MS–MS: Application to Screening New Adducts Formed from Acrylamide, Chroma, 72 (2010) 1043-1048.
[10] R.A. Kanaly, T. Hanaoka, H. Sugimura, H. Toda, S. Matsui, T. Matsuda, Development of the adductome approach to detect DNA damage in humans, Antioxidants & redox signaling, 8 (2006) 993-1001.
[11] E.E. Bessette, A.K. Goodenough, S. Langouet, I. Yasa, I.D. Kozekov, S.D. Spivack, R.J. Turesky, Screening for DNA adducts by data-dependent constant neutral loss-triple stage mass spectrometry with a linear quadrupole ion trap mass spectrometer, Anal Chem, 81 (2009) 809-819.
[12] B.H. Yun, T.A. Rosenquist, V. Sidorenko, C.R. Iden, C.H. Chen, Y.S. Pu, R. Bonala, F. Johnson, K.G. Dickman, A.P. Grollman, R.J. Turesky, Biomonitoring of aristolactam-DNA adducts in human tissues using ultra-performance liquid chromatography/ion-trap mass spectrometry, Chemical research in toxicology, 25 (2012) 1119-1131.
[13] P. Wang, D. Fisher, A. Rao, R.W. Giese, Nontargeted Nucleotide Analysis Based on Benzoylhistamine Labeling-MALDI-TOF/TOF-MS: Discovery of Putative 6-Oxo-Thymine in DNA, Analytical Chemistry, 84 (2012) 3811-3819.
[14] B. Van den Driessche, F. Lemiere, W. Van Dongen, E.L. Esmans, Alkylation of DNA by melphalan: investigation of capillary liquid chromatography-electrospray ionization tandem mass spectrometry in the study of the adducts at the nucleoside level, Journal of chromatography. B, Analytical technologies in the biomedical and life sciences, 785 (2003) 21-37.
[15] S. Balbo, S.S. Hecht, P. Upadhyaya, P.W. Villalta, Application of a High-Resolution Mass-Spectrometry-Based DNA Adductomics Approach for Identification of DNA Adducts in Complex Mixtures, Analytical Chemistry, 86 (2014) 1744-1752.

[16] S. Balbo, R.J. Turesky, P.W. Villalta, DNA adductomics, Chemical research in toxicology, 27 (2014) 356-366.
[17] R. Singh, F. Teichert, A. Seidel, J. Roach, R. Cordell, M.K. Cheng, H. Frank, W.P. Steward, M.M. Manson, P.B. Farmer, Development of a targeted adductomic method for the determination of polycyclic aromatic hydrocarbon DNA adducts using online column-switching liquid chromatography/tandem mass spectrometry, Rapid communications in mass spectrometry : RCM, 24 (2010) 2329-2340.
[18] N. Tretyakova, P.W. Villalta, S. Kotapati, Mass Spectrometry of Structurally Modified DNA, Chemical Reviews, 113 (2013) 2395-2436.
[19] G.G. Kuhnle, G.W. Story, T. Reda, A.R. Mani, K.P. Moore, J.C. Lunn, S.A. Bingham, Diet-induced endogenous formation of nitroso compounds in the GI tract, Free radical biology & medicine, 43 (2007) 1040-1047.
[20] G.G. Kuhnle, S.A. Bingham, Dietary meat, endogenous nitrosation and colorectal cancer, Biochemical Society transactions, 35 (2007) 1355-1357.
[21] L.J. Marnett, Lipid peroxidation-DNA damage by malondialdehyde, Mutation research, 424 (1999) 83-95.
[22] L.A. VanderVeen, M.F. Hashim, Y. Shyr, L.J. Marnett, Induction of frameshift and base pair substitution mutations by the major DNA adduct of the endogenous carcinogen malondialdehyde, Proc Natl Acad Sci U S A, 100 (2003) 14247-14252.
[23] L.J. Niedernhofer, J.S. Daniels, C.A. Rouzer, R.E. Greene, L.J. Marnett, Malondialdehyde, a product of lipid peroxidation, is mutagenic in human cells, The Journal of biological chemistry, 278 (2003) 31426-31433.
[24] A.L. Sesink, D.S. Termont, J.H. Kleibeuker, R. Van der Meer, Red meat and colon cancer: dietary haem-induced colonic cytotoxicity and epithelial hyperproliferation are inhibited by calcium, Carcinogenesis, 22 (2001) 1653-1659.
[25] B. Stewart, C.P. Wild, editors, World Cancer Report 2014 International Agency for Research on Cancer, WHO2014.
[26] A. Spor, O. Koren, R. Ley, Unravelling the effects of the environment and host genotype on the gut microbiome, Nat Rev Microbiol, 9 (2011) 279-290.
[27] T. Yatsunenko, F.E. Rey, M.J. Manary, I. Trehan, M.G. Dominguez-Bello, M. Contreras, M. Magris, G. Hidalgo, R.N. Baldassano, A.P. Anokhin, A.C. Heath, B. Warner, J. Reeder, J. Kuczynski, J.G. Caporaso, C.A. Lozupone, C. Lauber, J.C. Clemente, D. Knights, R. Knight, J.I. Gordon, Human gut microbiome viewed across age and geography, Nature, 486 (2012) 222-227.
[28] M.A. Hullar, A.N. Burnett-Hartman, J.W. Lampe, Gut microbes, diet, and cancer, Cancer Treat Res, 159 (2014) 377-399.
[29] S.P. Claus, H. Guillou, S. Ellero-Simatos, The gut microbiota: a major player in the toxicity of environmental pollutants?, Npj Biofilms And Microbiomes, 2 (2016) 16003.
[30] R.C. Massey, P.E. Key, A.K. Mallett, I.R. Rowland, An investigation of the endogenous formation of apparent total N-nitroso compounds in conventional microflora and germ-free rats, Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association, 26 (1988) 595-600.
[31] P. Louis, G.L. Hold, H.J. Flint, The gut microbiota, bacterial metabolites and colorectal cancer, Nat Rev Micro, 12 (2014) 661-672.
[32] S. Greenblum, P.J. Turnbaugh, E. Borenstein, Metagenomic systems biology of the human gut microbiome reveals topological shifts associated with obesity and inflammatory bowel disease, Proceedings of the National Academy of Sciences, 109 (2012) 594-599.
[33] G. Gottschalk, Bacterial metabolism, 2012.
[34] R. De Bont, N. van Larebeke, Endogenous DNA damage in humans: a review of quantitative data, Mutagenesis, 19 (2004) 169-185.

[35] M.H. Lewin, N. Bailey, T. Bandaletova, R. Bowman, A.J. Cross, J. Pollock, D.E.G. Shuker, S.A. Bingham, Red Meat Enhances the Colonic Formation of the DNA Adduct O6-Carboxymethyl Guanine: Implications for Colorectal Cancer Risk, Cancer Research, 66 (2006) 1859-1865.
[36] K. Stone, A. Uzieblo, L.J. Marnett, Studies of the reaction of malondialdehyde with cytosine nucleosides, Chemical research in toxicology, 3 (1990) 467-472.
[37] F.L. Chung, J. Pan, S. Choudhury, R. Roy, W. Hu, M.S. Tang, Formation of trans-4-hydroxy-2-nonenal- and other enal-derived cyclic DNA adducts from omega-3 and omega-6 polyunsaturated fatty acids and their roles in DNA repair and human p53 gene mutation, Mutation research, 531 (2003) 25-36.
[38] A. Besaratinia, G.P. Pfeifer, DNA adduction and mutagenic properties of acrylamide, Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 580 (2005) 31-40.
[39] M. Bignami, M. O'Driscoll, G. Aquilina, P. Karran, Unmasking a killer: DNA O6-methylguanine and the cytotoxicity of methylating agents, Mutation Research/Reviews in Mutation Research, 462 (2000) 71-82.
[40] R.L. Jirtle, M.K. Skinner, Environmental epigenomics and disease susceptibility, Nat Rev Genet, 8 (2007) 253-262.
[41] J.M. Zingg, P.A. Jones, Genetic and epigenetic aspects of DNA methylation on genome expression, evolution, mutation and carcinogenesis, Carcinogenesis, 18 (1997) 869-882.
[42] J. Collier, Epigenetic regulation of the bacterial cell cycle, Curr Opin Microbiol, 12 (2009) 722-729.
[43] R.A. Kanaly, S. Matsui, T. Hanaoka, T. Matsuda, Application of the adductome approach to assess intertissue DNA damage variations in human lung and esophagus, Mutation research, 625 (2007) 83-93.
[44] R.A. Kanaly, R. Micheletto, T. Matsuda, Y. Utsuno, Y. Ozeki, N. Hamamura, Application of DNA adductomics to soil bacterium Sphingobium sp. strain KK22, MicrobiologyOpen, 4 (2015) 841-856.
[45] K. Kato, E. Yamamura, M. Kawanishi, T. Yagi, T. Matsuda, A. Sugiyama, Y. Uno, Application of the DNA adductome approach to assess the DNA-damaging capability of in vitro micronucleus test-positive compounds, Mutation research, 721 (2011) 21-26.
[46] B. Spilsberg, T. Rundberget, L.E. Johannessen, A.B. Kristoffersen, A. Holst-Jensen, K.G. Berdal, Detection of Food-Derived Damaged Nucleosides with Possible Adverse Effects on Human Health Using a Global Adductomics Approach, Journal of Agricultural and Food Chemistry, 58 (2010) 6370-6375.
[47] D. Compagnone, R. Curini, G. D'Ascenzo, M. Del Carlo, C. Montesano, S. Napoletano, M. Sergi, Neutral loss and precursor ion scan tandem mass spectrometry for study of activated benzopyrene-DNA adducts, Analytical and bioanalytical chemistry, 401 (2011) 1983-1991.
[48] T. Matsuda, H. Tao, M. Goto, H. Yamada, M. Suzuki, Y. Wu, N. Xiao, Q. He, W. Guo, Z. Cai, N. Kurabe, K. Ishino, Y. Matsushima, K. Shinmura, H. Konno, M. Maekawa, Y. Wang, H. Sugimura, Lipid peroxidation-induced DNA adducts in human gastric mucosa, Carcinogenesis, 34 (2013) 121-127.
[49] P.-H. Chou, S. Kageyama, S. Matsuda, K. Kanemoto, Y. Sasada, M. Oka, K. Shinmura, H. Mori, K. Kawai, H. Kasai, H. Sugimura, T. Matsuda, Detection of Lipid Peroxidation-Induced DNA Adducts Caused by 4-Oxo-2(E)-nonenal and 4-Oxo-2(E)-hexenal in Human Autopsy Tissues, Chemical research in toxicology, 23 (2010) 1442-1448.
[50] Y. Ishii, S. Takasu, K. Kuroda, K. Matsushita, A. Kijima, T. Nohmi, K. Ogawa, T. Umemura, Combined application of comprehensive analysis for DNA modification and reporter gene mutation assay to evaluate kidneys of gpt delta rats given madder color or its constituents, Analytical and bioanalytical chemistry, 406 (2014) 2467-2475.

[51] K. Ishino, T. Kato, M. Kato, T. Shibata, M. Watanabe, K. Wakabayashi, H. Nakagama, Y. Totsuka, Comprehensive DNA adduct analysis reveals pulmonary inflammatory response contributes to genotoxic action of magnetite nanoparticles, International journal of molecular sciences, 16 (2015) 3474-3492.
[52] J.M. Gregson, J.A. McCloskey, Collision-induced dissociation of protonated guanine, International Journal of Mass Spectrometry and Ion Processes, 165–166 (1997) 475-485.
[53] M. Goggin, R. Loeber, S. Park, V. Walker, J. Wickliffe, N. Tretyakova, HPLC-ESI+-MS/MS analysis of N7-guanine-N7-guanine DNA cross-links in tissues of mice exposed to 1,3-butadiene, Chemical research in toxicology, 20 (2007) 839-847.
[54] J. Vanden Bussche, S.A. Moore, F. Pasmans, G.G. Kuhnle, L. Vanhaecke, An approach based on ultra-high pressure liquid chromatography-tandem mass spectrometry to quantify O6-methyl and O6-carboxymethylguanine DNA adducts in intestinal cell lines, Journal of chromatography. A, 1257 (2012) 25-33.
[55] P. Iannitti-Tito, A. Weimann, G. Wickham, M.M. Sheil, Structural analysis of drug-DNA adducts by tandem mass spectrometry, Analyst, 125 (2000) 627-634.
[56] A.K. Chaudhary, G.R. Reddy, I.A. Blair, L.J. Marnett, Characterization of an N6-oxopropenyl-2'-deoxyadenosine adduct in malondialdehyde-modified DNA using liquid chromatography/electrospray ionization tandem mass spectrometry, Carcinogenesis, 17 (1996) 1167-1170.
[57] G. Nauwelaers, E.E. Bessette, D. Gu, Y. Tang, J. Rageul, V. Fessard, J.M. Yuan, M.C. Yu, S. Langouet, R.J. Turesky, DNA adduct formation of 4-aminobiphenyl and heterocyclic aromatic amines in human hepatocytes, Chemical research in toxicology, 24 (2011) 913-925.
[58] K.E. Pietsch, P.M. van Midwoud, P.W. Villalta, S.J. Sturla, Quantification of acylfulvene- and illudin S-DNA adducts in cells with variable bioactivation capacities, Chemical research in toxicology, 26 (2013) 146-155.
[59] E.E. Bessette, S.D. Spivack, A.K. Goodenough, T. Wang, S. Pinto, F.F. Kadlubar, R.J. Turesky, Identification of carcinogen DNA adducts in human saliva by linear quadrupole ion trap/multistage tandem mass spectrometry, Chemical research in toxicology, 23 (2010) 1234-1244.
[60] P. Wang, J. Gao, G. Li, O. Shimelis, R.W. Giese, Nontargeted analysis of DNA adducts by mass-tag MS: reaction of p-benzoquinone with DNA, Chemical research in toxicology, 25 (2012) 2737-2743.
[61] B. Van den Driessche, W. Van Dongen, F. Lemière, E.L. Esmans, Implementation of data-dependent acquisitions in the study of melphalan DNA adducts by miniaturized liquid chromatography coupled to electrospray tandem mass spectrometry, Rapid Communications in Mass Spectrometry, 18 (2004) 2001-2007.
[62] G.L. Glish, D.J. Burinsky, Hybrid mass spectrometers for tandem mass spectrometry, J Am Soc Mass Spectrom, 19 (2008) 161-172.
[63] L.Y. Hemeryck, A.I. Decloedt, J. Vanden Bussche, K.P. Geboes, L. Vanhaecke, High resolution mass spectrometry based profiling of diet-related deoxyribonucleic acid adducts, Anal Chim Acta, 892 (2015) 123-131.
[64] R.A. Zubarev, A. Makarov, Orbitrap Mass Spectrometry, Analytical Chemistry, 85 (2013) 5288-5296.
[65] S. Cristoni, L.R. Bernardi, Development of new methodologies for the mass spectrometry study of bioorganic macromolecules, Mass Spectrom Rev, 22 (2003) 369-406.
[66] K.L. Randall, D. Argoti, J.D. Paonessa, Y. Ding, Z. Oaks, Y. Zhang, P. Vouros, An improved liquid chromatography-tandem mass spectrometry method for the quantification of 4-aminobiphenyl DNA adducts in urinary bladder cells and tissues, Journal of chromatography. A, 1217 (2010) 4135-4143.

[67] J.R. Soglia, R.J. Turesky, A. Paehler, P. Vouros, Quantification of the heterocyclic aromatic amine DNA adduct N-(deoxyguanosin-8-yl)-2-amino-3-methylimidazo[4,5-f]quinoline in livers of rats using capillary liquid chromatography/microelectrospray mass spectrometry: a dose-response study, Anal Chem, 73 (2001) 2819-2827.
[68] J. Embrechts, F. Lemiere, W. Van Dongen, E.L. Esmans, P. Buytaert, E. Van Marck, M. Kockx, A. Makar, Detection of estrogen DNA-adducts in human breast tumor tissue and healthy tissue by combined nano LC-nano ES tandem mass spectrometry, J Am Soc Mass Spectrom, 14 (2003) 482-491.
[69] D.R. Stoll, X. Li, X. Wang, P.W. Carr, S.E.G. Porter, S.C. Rutan, Fast, comprehensive two-dimensional liquid chromatography, Journal of Chromatography A, 1168 (2007) 3-43.
[70] C. Zhao, Z. Wu, G. Xue, J. Wang, Y. Zhao, Z. Xu, D. Lin, G. Herbert, Y. Chang, K. Cai, G. Xu, Ultra-high capacity liquid chromatography chip/quadrupole time-of-flight mass spectrometry for pharmaceutical analysis, Journal of Chromatography A, 1218 (2011) 3669-3674.
[71] G.L. Gauthier, R. Grimm, Miniaturization: Chip-based liquid chromatography and proteomics, Drug discovery today. Technologies, 3 (2006) 59-66.
[72] A.D. Bani-Yaseen, T. Kawaguchi, A.K. Price, C.T. Culbertson, R. Jankowiak, Integrated microfluidic device for the separation and electrochemical detection of catechol estrogen-derived DNA adducts, Analytical and bioanalytical chemistry, 399 (2011) 519-524.
[73] J.K. Nicholson, E. Holmes, J.M. Kinross, A.W. Darzi, Z. Takats, J.C. Lindon, Metabolic phenotyping in clinical and surgical environments, Nature, 491 (2012) 384-392.
[74] K.W. Jordan, J. Nordenstam, G.Y. Lauwers, D.A. Rothenberger, K. Alavi, M. Garwood, L.L. Cheng, Metabolomic characterization of human rectal adenocarcinoma with intact tissue magnetic resonance spectroscopy, Diseases of the colon and rectum, 52 (2009) 520-525.
[75] S. Patel, S. Ahmed, Emerging field of metabolomics: big promise for cancer biomarker identification and drug discovery, J Pharm Biomed Anal, 107 (2015) 63-74.
[76] Y. Zhang, Y. Liu, L. Li, J. Wei, S. Xiong, Z. Zhao, High resolution mass spectrometry coupled with multivariate data analysis revealing plasma lipidomic alteration in ovarian cancer in Asian women, Talanta, 150 (2016) 88-96.
[77] C. Rombouts, L.Y. Hemeryck, T. Van Hecke, S. De Smet, W.H. De Vos, L. Vanhaecke, Scientific Reports, *accepted*, (2017).
[78] N. Blow, Metagenomics: Exploring unseen communities, Nature, 453 (2008) 687-690.
[79] T.J. Key, A. Schatzkin, W.C. Willett, N.E. Allen, E.A. Spencer, R.C. Travis, Diet, nutrition and the prevention of cancer, Public Health Nutr, 7 (2004) 187-200.
[80] E. Vogtmann, J.J. Goedert, Epidemiologic studies of the human microbiome and cancer, British journal of cancer, 114 (2016) 237-242.
[81] T. Irrazábal, A. Belcheva, Stephen E. Girardin, A. Martin, Dana J. Philpott, The Multifaceted Role of the Intestinal Microbiota in Colon Cancer, Molecular Cell, 54 (2014) 309-320.
[82] L. Blanchet, A. Smolinska, Data Fusion in Metabolomics and Proteomics for Biomarker Discovery, Methods in molecular biology (Clifton, N.J.), 1362 (2016) 209-223.
[83] L. Blanchet, A. Smolinska, A. Attali, M.P. Stoop, K.A. Ampt, H. van Aken, E. Suidgeest, T. Tuinstra, S.S. Wijmenga, T. Luider, L.M. Buydens, Fusion of metabolomics and proteomics data for biomarkers discovery: case study on the experimental autoimmune encephalomyelitis, BMC Bioinformatics, 12 (2011) 1-12.
[84] W. Nie, L. Yan, Y.H. Lee, C. Guha, I.J. Kurland, H. Lu, Advanced mass spectrometry-based multi-omics technologies for exploring the pathogenesis of hepatocellular carcinoma, Mass Spectrometry Reviews, 35 (2016) 331-349.

Summary

Epidemiological research has demonstrated that the consumption of red and processed meat significantly contributes to the risk of developing colorectal cancer (CRC). The exact underlying cause has not been fully elucidated, but several hypotheses have been put forward, aiming to explain this causal relationship. At the time, the main red meat-CRC hypothesis is the heme hypothesis, which is based on the fact that red, but not white meat consumption, has been linked to CRC, and that red meat like e.g. beef contains more heme iron than white meat like e.g. chicken. More specifically, the heme hypothesis states that the ingestion and digestion of heme iron stimulates the formation of N-nitroso compounds (NOCs) and lipid peroxidation products (LPOs). Both NOCs and LPOs can exert geno- as well as cytotoxic effects, and as such contribute to carcinogenesis. The research conducted within the framework of this doctoral thesis aimed to further unravel the carcinogenic effects of red meat consumption, primarily focusing on NOC and LPO induced DNA adduct formation. This was addressed in 7 different chapters.

Chapter I – The first chapter of this dissertation contains a general introduction to diet-related carcinogenesis, discussing facts, figures, causes and consequences. This is followed by a clarification of the concept of the DNA adductome, and its position in relation to the exposome. Next, an overview of the current knowledge on diet-related DNA adduct formation in relation to carcinogenesis is given, further highlighting the use of targeted as well as untargeted DNA adduct research to help unravel the pathways underlying diet-related, and more specifically, meat-related carcinogenesis. Hence, an overview of the current hypotheses on red and processed meat related carcinogenesis is provided, furthermore explaining the potential role of DNA adduct formation. Looking ahead, important practical considerations for DNA adduct research are discussed briefly, after which the specific focus and aim of this PhD is revealed.

Chapter II – In chapter II, the effects of red meat digestion on cell viability, gastro-intestinal metabolic activity, NOC formation, DNA alkylation and lipid peroxidation are investigated. To this purpose, the digestion of beef, pork and chicken were simulated *in vitro*, after which (1) the cytotoxic effects of different meat digests were assessed by means of a cell proliferation assay, (2) metabolic activity was assessed *via* short chain fatty acid analysis, (3) the formation of NOCs was measured by analysis of Apparent Total N-nitroso Compounds, (4) the malondialdehyde (MDA) content of meat digests was measured using the thiobarbituric acid reactive substances assay, and (5) DNA alkylation was determined by assessment of O^6-carboxymethylguanine (O^6-CMG) formation. The results demonstrate that the cytotoxicity and metabolic activity of beef, pork and chicken digests did not significantly differ. In contrast, the digestion of beef, a model for red meat, resulted in the increased formation of O^6-CMG and MDA in comparison to the digestion of pork and chicken. The formation of O^6-CMG furthermore appeared to be higher in pork digests compared to chicken digests, hinting at a heme iron induced dose-response effect. As such, it could be concluded that red meat digestion significantly contributes to DNA alkylation (reflected by O^6-CMG DNA adduct formation) and lipid peroxidation although the gastro-

intestinal formation of O^6-CMG strongly differed between individuals. In fact, it could be confirmed that the microbiota actively contributes to O^6-CMG formation, which is a clear reflection of the interfering role of the gut microbiome in health and disease.

Chapter III – Since a top-down DNA adductomics approach can facilitate the assessment of environmental exposure to genotoxic chemicals, a DNA adductomics methodology was developed to enable in-depth investigation of diet-, and more specifically, NOC- and LPO-related DNA adduct formation. To this purpose, an in-house diet-related DNA adduct database, containing known alkylation as well as oxidation induced DNA adduct types, and a high resolution mass spectrometry (HRMS) based methodology were established. After optimization, the method proved to be specific, sensitive, selective, precise and true, whilst good to excellent linearity could also be confirmed. The *in vitro* and *in vivo* application of the described DNA adductomics methodology and workflow facilitated targeted (i.e. O^6-CMG, O^6-methylguanine, pyrimido[1,2-a]purin-10(1H)-one and α-methyl-γ-hydroxy-1,N_2-propanoguanine) as well as untargeted DNA adduct detection and profiling, offering highly promising results and future prospects. The DNA adduct database expedited tentative DNA adduct identification and data interpretation. At first, the in-house diet-related DNA adduct database contained 123 different DNA adduct types, but the database is in fact updated continuously, allowing up-to-date and top-notch research.

Chapter IV – Following the development of a HRMS-based DNA adductomics methodology, DNA adduct formation as a result of red *vs.* white meat digestion could be investigated more in-depth. In chapter IV, the results from 2 different experimental setups are described. The first experimental setup included DNA adductome mapping of beef digests obtained by the *in vitro* simulation of (colonic) digestion using the fecal inocula of 5 healthy volunteers. The results revealed a strong interindividual variability with regard to the types and levels of DNA adduct formation. In addition, it could be observed that some DNA adduct types originated from the fecal inoculum (due to *in vivo* occurrence/formation), but were not actively formed during *in vitro* beef digestion, whilst others significantly increased during colonic beef digestion and/or only appeared after completion of colonic meat digestion. The second experimental setup focused on differences in MDA and DNA adduct formation upon red *vs.* white meat digestion, using the repeatedly collected fecal inocula of 2 selected volunteers. By means of the supplementation of calcium carbonate ($CaCO_3$) to meat preparations, the possible interfering role of calcium, which is hypothesized to have CRC-protective attributes, on MDA and DNA adduct formation was assessed as well. The obtained results confirmed the earlier reported finding that red meat digestion stimulates MDA and O^6-CMG DNA adduct formation. $CaCO_3$ supplementation resulted in both toxic and anti-toxic effects; i.e. stimulation of O^6-CMG production, but reduction of MDA formation. The mapped DNA adduct profile differed according to digested meat type, uncovering different putative DNA adducts associated with digestion of beef or chicken with or without supplemented $CaCO_3$. More specifically, the formamidopyrimidine-

adenine DNA adduct was found to be discriminative for meat digests without added $CaCO_3$, whilst carboxyethylcytosine was significantly higher in beef digests, and methoxymethylcytosine (or its hydroxyethylcytosine isomer) was found to be lower in meat digests supplemented with $CaCO_3$. These findings suggest that the gastrointestinal digestion of red meat indeed stimulates the formation of genotoxic metabolites, which can moreover be modulated by calcium.

Chapter V – The work described in chapter V is a continuation of the work outlined in chapter IV. More specifically, chapter V focusses on *in vitro* DNA adductome mapping of red *vs.* white meat digests, but on a much larger scale; i.e. using the fecal inocula of 10 healthy volunteers. Additional experiments encompassing the digestion of myoglobin, the heme-containing meat protein, allowed more in-depth assessment of the genotoxic effects of heme-rich meat digestion. In total, 90 DNA adduct types could be (tentatively) identified, encompassing several known alkylation and (lipid per)oxidation induced DNA adducts like O^6- and other methylguanines, O^6-CMG, methylthymine, ethylthymine, hydroxymethylhydantion, etc. The results demonstrate a significant interindividual variability, but for the vast majority, the observed DNA adduct levels increased during colonic meat digestion, suggesting active formation by the colonic microbiota. More importantly, a significantly more pronounced formation of 26 DNA adduct types, including e.g. O^6-CMG and ethylthymine, could be observed upon red meat digestion compared to white meat digestion. More specifically, it could be observed that (1) the levels of hydroxymethylhydantion and a triple malondialdehyde cytosine (M_3C) adduct, which are both oxidatively induced nucleobase alterations, were significantly higher after small bowel beef digestion (in comparison to chicken), and (2) 3 DNA adduct types; i.e. hydroxyethylthymine (or methoxymethylthymine), carboxyethylthymine, and 3,N^4-ethenocytosine, demonstrated a significantly increased formation upon myoglobin addition. Multivariate statistics furthermore revealed 4 additional beef digestion markers; methyl-, ethyl-, hydroxymethyl-, and tetramethylthymine. As a result, those specific alkylation and/or oxidation induced DNA adduct types were singled out as potential heme-rich meat digestion markers, which is in support of the heme, NOC and LPO hypothesis. It furthermore confirms that DNA adduct formation may indeed contribute to red meat related CRC risk.

Chapter VI – This chapter describes the investigation of shifts in the DNA adductome of rats due to red *vs.* white meat consumption and digestion. 24 Sprague-Dawley rats were divided into 4 randomly composed groups and either fed a low or high fat beef diet, or a low or high fat chicken diet during 14 consecutive days, after which the DNA adductome of liver, duodenum and colon were mapped. Untargeted DNA adduct analysis revealed a distinctly different DNA adduct profile in liver, duodenum and colon. Several DNA adduct types appeared to be significantly different in tissue of rats that were fed a different meat based diet. The results yielded a list of 22 putative DNA adducts of interest; trihydroxybutyl-U, carboxyl-A, methyl-C, oxohexenal-C, 1,N_2-propano-G, nitro-C, 2 isomers of malondialdehyde-2x-acetaldehyde-A, malondialdehyde-2x-G, 2 isomers of hydroxyhydro-C, heptenal-G, hydroxyethyl-C (or

methoxymethyl-C) and carbamoylhydroxyethyl-G DNA adducts appeared to be higher in beef fed rats when compared to chicken fed rats; hydroxybutyl-A, hydroxymethyl-A (or methyl-G or methoxy-A) and hydroxybutyl-G increased due to the daily consumption of a meat preparation with added lard; and a carboxyl-A isomer, a crotonaldehyde-G isomer, carboxymethyl-G (or glyoxal-G), carboxyethyl-G (or carboxyhydroxyethyl-A or methylglyoxal-G) and a malondialdehyde-2x-acetaldehyde-A isomer appeared to be significantly higher after the daily consumption and digestion of beef as well as lard. Yet again, these findings suggest that the gastrointestinal digestion of red meat stimulates the formation of genotoxic metabolites, resulting in the formation of alkylation and/or oxidation induced DNA adducts. In addition, it was demonstrated that dietary fat can induce shifts in the DNA adductome as well.

Chapter VII – The final chapter of this dissertation summarizes and discusses the most prominent findings of this doctoral thesis, primarily focusing on the successful development of a DNA adductomics methodology and the in-depth profiling of diet-induced shifts in the *in vitro* and *in vivo* DNA adductome. Taking into account all obtained *in vivo* and *in vitro* results, 7 DNA adduct types, including O^6-CMG, dimethyl- or ethyl-T, methyl-G, malondialdehyde-2x-G, heptanal-G and malondialdehyde-3x-C (M_3C) could be singled out as potential red meat digestion biomarkers. This is highly relevant to the red meat-CRC hypothesis because the formation of these DNA adduct types can be traced back to alkylation and/or oxidation of DNA by e.g. NOCs and/or LPOs formed during red meat digestion. Increased lipid peroxidation due to the red meat digestion could be confirmed on multiple occasions, but the hypothesized origin of DNA alkylation (i.e. NOCs) could not be identified. Therefore, follow-up research to further unravel the role of DNA adduct formation in the red meat-CRC pathway, and the mutagenic potential and human *in vivo* relevance of these particular DNA adduct types is highly recommended. In this context, the implementation of complimentary omics techniques would furthermore be highly beneficial, allowing more in-depth (follow-up) CRC-research. In particular, fusion of DNA adductomics, metagenomics and metabolomics offers favorable future prospects. At the time, the field of DNA addductomics encounters 2 major bottlenecks due restrictions with regard to DNA sample size and confident DNA adduct identification. Improvement of (the use of) hybrid HRMS as well as implementation of chromatographic innovations may however provide a remedy in the near future.

Samenvatting

Epidemiologisch onderzoek heeft aangetoond dat de consumptie van rood en verwerkt vlees significant bijdraagt tot het risico op de ontwikkeling van dikke darmkanker (DDK). De onderliggende oorzaak werd nog niet volledig opgehelderd, hoewel er, over de jaren heen, reeds verschillende verklarende hypotheses naar voor geschoven werden. De 'haemhypothese' kan vandaag de dag op de grootste bijval rekenen. Deze hypothese steunt op het feit dat de consumptie van rood, maar niet wit vlees gelinkt wordt aan de ontwikkeling van DDK en dat rood vlees, zoals bv. rundvlees, beduidend meer haem bevat dan wit vlees, zoals bv. kip. De opname van haem zou ervoor zorgen dat de endogene vorming van N-nitrosoverbindingen (NOC's) en vetperoxidatieproducten (VPO's) in het gastro-intestinaal stelsel gestimuleerd wordt. Aangezien zowel NOC's als VPO's geno- en cytotoxische effecten kunnen uitoefenen, kan dit proces mogelijk bijdragen tot de ontwikkeling van kanker. In het kader van dit doctoraat werd onderzoek verricht naar de mogelijke carcinogene effecten van de consumptie van rood vlees. Hierbij werd voornamelijk gefocust op de opsporing van NOC en VPO gerelateerde DNA-schade, meer bepaald DNA-adducten. Dit alles werd uitgewerkt en neergeschreven in 7 verschillende hoofdstukken.

Hoofdstuk I – Het eerste hoofdstuk van deze doctoraatsthesis bevat een algemene introductie over dieetgerelateerde carcinogenese. Dit wordt onder meer uitgelegd aan de hand van enkele belangrijke feiten en cijfers, met bijzondere aandacht voor de mogelijke oorzaken en gevolgen van kanker. Wat volgt, is een uitdieping van de term 'DNA adductomics' en de situering van het DNA-adductoom in en ten opzichte van het exposoom. Hierna wordt een overzicht gegeven van alle huidige kennis omtrent de vorming van DNA-adducten onder invloed van de consumptie van bepaalde voedingsmiddelen en de associatie met de ontwikkeling van kanker. Het belang van DNA-adducten in onderzoek naar dieetgerelateerde, en dan vooral vleesconsumptiegerelateerde carcinogenese wordt hierbij gedemonstreerd. Om dit doctoraatsonderzoek duidelijk te situeren, wordt vervolgens een overzicht gegeven van alle huidige hypotheses omtrent de ontwikkeling van DDK ten gevolge van de consumptie van rood en verwerkt vlees. In deze context wordt het belang van DNA-adductonderzoek nogmaals verduidelijkt en wordt er tevens aandacht geschonken aan de praktische noden van onderzoek naar de vorming van DNA-adducten. Finaal wordt de specifieke focus en het doel van dit doctoraatsonderzoek uitgelijnd.

Hoofdstuk II – In hoofdstuk II wordt het effect van de consumptie van rood vlees op celviabiliteit, gastro-intestinale metabole activiteit, de vorming van NOC's, alkylatie van DNA en vetperoxidatie onderzocht. Hiertoe werd de vertering van rundvlees, varkensvlees en kip gesimuleerd onder *in vitro* omstandigheden, zodoende de invloed van de bekomen vleesdigesten op (1) cytotoxiciteit, (2) gastro-intestinale metabolische activiteit onder de vorm van de productie van korteketenvetzuren, (3) de vorming van NOC's, door middel van bepaling van 'Apparent Total N-nitroso Compounds', (4) de vorming van het vetperoxidatieproduct malondialdehyde (MDA) en (5) de alkylatie van DNA onder de vorm van de productie van O^6-

carboxymethylguanine (O^6-CMG) na te gaan. De resultaten van deze analyses toonden aan dat de vertering van het kip, varkens- of rundvlees geen aanleiding gaf tot significante verschillen in cytotoxiciteit en korteketenvetzuurproductie. Daarentegen gaf de vertering van rund, als model voor rood vlees, wel aanleiding tot een verhoogde aanmaak van O^6-CMG en MDA in vergelijking met varken of kip. De vorming van O^6-CMG bleek bovendien hoger onder invloed van de vertering van varkensvlees in vergelijking met kip, wat een haemijzer gerelateerde dosis-respons relatie suggereert. Er kon dan ook besloten worden dat de vertering van rood vlees significant bijdraagt tot de alkylatie van DNA (onder de vorm van O^6-CMG-productie) en vetperoxidatie, hoewel de vorming van O^6-CMG sterk persoonsgebonden is. Daarenboven kon vastgesteld worden dat het fecaal microbioom actief bijdraagt aan de vorming van O^6-CMG, een reflectie van het feit dat het gastro-intestinaal microbioom een belangrijke rol speelt in zowel ziekte als gezondheid.

Hoofdstuk III – Onderzoek naar de vorming van DNA-adducten op een niet-gerichte manier, nl. met behulp van DNA-adductomics, kan een grote bijdrage betekenen voor de correcte inschatting van de blootstelling aan genotoxische verbindingen in ons leefmilieu. In deze context beschrijft hoofdstuk III de ontwikkeling, validatie en toepassing van een DNA-adductomics methodologie die een diepgaander onderzoek naar de vorming van NOC- en VPO-gerelateerde DNA-adducten in relatie tot het dieet mogelijk maakt. Meer specifiek werd hiertoe een database met voedingsgerelateerde DNA-adducten opgesteld en een analytische methode ontwikkeld die gebruik maakt van hoge resolutie massaspectrometrie (HRMS). De HRMS-methode werd geoptimaliseerd en gevalideerd om een voldoende hoge specificiteit, gevoeligheid, selectiviteit, juistheid en precisie te garanderen. De beschreven methodologie creëert de mogelijkheid om DNA-adducten zowel op een gerichte (omvat de detectie van O^6-CMG, O^6-methylguanine, pyrimido[1,2-a]purin-10(1H)-one en α-methyl-γ-hydroxy-1,N_2-propanoguanine) als ongerichte manier te detecteren. Een demonstratie van de *in vitro* en *in vivo* toepassing leverde veelbelovende resultaten en toekomstperspectieven. Het gebruik van de eigen DNA-adductdatabase vergemakkelijkte daarenboven de DNA-adductidentificatie en data-interpretatie. Initieel bevatte de database zo'n 123 verschillende dieetgerelateerde DNA-adducten. Sindsdien wordt de database voortdurend bijgewerkt om te allen tijde kwaliteitsvol onderzoek af te leveren.

Hoofdstuk IV – Na de ontwikkeling van een HRMS DNA-adductomics methodologie, kon een diepgaander onderzoek naar de vorming van DNA-adducten ten gevolge van de vertering van rood *vs.* wit vlees aangevat worden. In deze context omschrijft hoofdstuk IV de uitwerking van 2 verschillende experimenten. In het eerste experiment werd het DNA-adductoom van rundvleesdigesten in kaart gebracht, gebruik makende van de fecale inocula van 5 gezonde vrijwilligers. Dit toonde aan dat het gastro-intestinaal DNA-adductoom een sterke interindividuele variatie vertoont; de detectie van welbepaalde DNA-adducten kon teruggeleid worden naar hun aanwezigheid in het fecaal inoculum (door de natuurlijke aanwezigheid en/of vorming in het lichaam), terwijl andere types DNA-adducten pas gedetecteerd konden worden

na simulatie van de vertering in de dikke darm. Afhankelijk van het type DNA-adduct, leidde de *in vitro* vertering van rundsvlees tot een concentratiestijging of -daling. Het tweede experiment maakte het mogelijk om de verschillen in vetperoxidatie en DNA-adductproductie bij de vertering van rood en wit vlees te vergelijken, gebruik makende van de fecale inocula van 2 gezonde vrijwilligers. Door de supplementatie van vlees met calciumcarbonaat ($CaCO_3$) kon bovendien een inschatting gemaakt worden van de interfererende invloed van calcium, wat van belang is omdat de consumptie van calcium reeds gelinkt werd met een verlaagd risico op DDK. De resultaten van dit experiment toonden wederom aan dat de vertering van rood vlees (in vergelijking met de vertering van wit vlees) aanleiding geeft tot een toename van MDA en O^6-CMG. De toevoeging van $CaCO_3$ aan vleesbereidingen resulteerde daarentegen in een reductie van de productie van MDA, maar een verhoogde productie van O^6-CMG. Zoals verwacht, wijzigde ook het DNA-adductoom naargelang het type vlees dat verteerd werd. Bepaalde types DNA-adducten konden hierbij gelinkt worden aan de vertering van rund of kip, of het feit of $CaCO_3$ al dan niet toegevoegd werd aan de vleesbereiding. Meer specifiek bleek carboxyethylcytosine significant verhoogd na de vertering van rood vlees, terwijl de formamidopyrimidine-adenine en methoxymethylcytosine (of z'n hydroxyethylcytosine isomer) DNA-adducten beduidend frequenter aangetroffen konden worden in vleesdigesten zonder toegevoegd $CaCO_3$. Deze bevindingen tonen aan dat de vertering van rood vlees aanleiding kan geven tot de vorming van genotoxische verbindingen en dat calcium deze effecten kan moduleren.

Hoofdstuk V – In hoofdstuk V wordt het werk uit hoofdstuk IV verdergezet; wijzigingen in het *in vitro* gastro-intestinaal DNA adductoom ten gevolge van de vertering van rood *vs.* wit vlees wordt verder in kaart gebracht, dit keer gebruik makende van de fecale inocula van 10 gezonde vrijwilligers. Een bijkomend experiment met de toevoeging van myoglobine, het vleeseigen proteïne die de haemmolecule bevat, liet daarenboven toe om de genotoxische effecten van vlees rijk aan haem, diepgaander te bestuderen. In totaal konden 90 DNA-adducttypes, waaronder verschillende alkylatie- en oxidatie-geïnduceerde DNA-adducten zoals O^6- en andere methylguanines, O^6-CMG, methylthymine, ethylthymine, hydroxymethylhydantion, etc., gedetecteerd worden. Opnieuw vertonen de resultaten een uitgesproken interindividuele variabiliteit. De grote meerderheid van de gedetecteerde DNA-adducten vertoonde een stijging na (gesimuleerde) dikke darmvertering, wat een actieve vorming door de dikke darmmicrobiota doet vermoeden. Van groot belang is de detectie van 26 DNA-adducttypes, waaronder o.a. O^6-CMG en ethylthymine, die beduidend hogere concentraties vertoonden bij de vertering van rood vlees in vergelijking met de vertering van wit vlees. Zo kon onder meer vastgesteld worden dat (1) de concentratie van het hydroxymethylhydantion DNA-adduct en een MDA-geïnduceerd cytosine adduct (M_3C), beiden het indirect resultaat van (vetper)oxidatie, significant hoger waren in digesten van rood vlees (in vergelijking met wit vlees) na de dunne darmsimulatie en (2) de vorming van 3 andere DNA-adducten, met name hydroxyethylthymine (of methoxymethylthymine), carboxyethylthymine, en 3,N^4-ethenocytosine, significant steeg onder invloed van de aanwezigheid van toegevoegd myoglobine. Daarnaast kon multivariate statistiek aantonen dat digesten van rood vlees onderscheiden kunnen worden van de digesten van wit

vlees op basis van de productie van methyl-, ethyl-, hydroxymethyl-, en tetramethylthymine. Bijgevolg konden bovenstaande DNA-adducten opgelijst worden als mogelijke biomerkers voor de vertering van rood vlees. Gezien hun vorming resulteert uit de alkylatie en/of oxidatie van DNA, ondersteunen deze bevindingen de NOC-, VPO- en haemhypothese, waardoor kan besloten worden dat de vorming van DNA-adducten weldegelijk een rol kan spelen bij de ontwikkeling van DDK.

Hoofdstuk VI – Dit hoofdstuk beschrijft de studie van de wijzigingen in het DNA-adductoom van ratten onder invloed van de opname en vertering van rood *vs.* wit vlees. 24 ratten (Sprague-Dawley) werden willekeurig ingedeeld in 4 verschillende groepen die een vast dieet met kip of rund, al dan niet met toegevoegd vet, gevoederd werden. Het dieet werd aangehouden gedurende 14 dagen, waarna lever-, duodenum- en colonstalen verzameld werden om het DNA-adductoom in kaart te brengen. DNA adductomics onthulde dat het lever-, duodenum- en colonweefsel een significant verschillend DNA-adductprofiel vertoonden. Bovendien bleek het DNA-adductoom significant verschillend bij ratten die een ander dieet kregen. Maar liefst 22 DNA-adducten bleken van bijzonder belang te zijn, waaronder trihydroxybutyl-U, carboxyl-A, methyl-C, oxohexenal-C, 1,N_2-propano-G, nitro-C, 2 malondialdehyde-2x-acetaldehyde-A isomeren, malondialdehyde-2x-G, 2 hydroxyhydro-C isomeren, heptenal-G, hydroxyethyl-C (or methoxymethyl-C) en carbamoylhydroxyethyl-G die significant frequenter voorkwamen in de weefsels van ratten die gevoederd werden met rundsvlees in vergelijking met ratten die kip te eten kregen. De concentraties van 3 andere DNA-adducten, nl. hydroxybutyl-A, hydroxymethyl-A (of methyl-G of methoxy-A) en hydroxybutyl-G, namen significant toe onder invlooed van de dagdagelijkse consumptie van extra dierlijk vet, terwijl een carboxyl-A isomeer, een crotonaldehyde-G isomeer, carboxymethyl-G (of glyoxal-G), carboxyethyl-G (of carboxyhydroxyethyl-A of methylglyoxal-G) en een malondialdehyde-2x-acetaldehyde-A isomeer beduidend hogere concentraties vertoonden na de consumptie van zowel rund als toegevoegd dierlijk vet (in vergelijking met een dieet met kip of een vetarm dieet). Dit alles bevestigt dat de vertering van rood vlees de vorming van genotoxische verbindingen in de hand kan werken, met de mogelijke alkylatie en/of oxidatie van DNA tot gevolg. Bovendien lijkt niet alleen het type vlees dit proces te beïnvloeden, maar ook de hoeveelheid geconsumeerd dierlijk vet.

Hoofdstuk VII – Het laatste hoofdstuk van dit proefschrift vat alle bekomen resultaten samen en bespreekt de belangrijkste bevindingen. De focus ligt hierbij op de ontwikkeling van een DNA adductomics methodologie en de uitgebreide profilering van wijzigingen in het *in vitro* en *in vivo* DNA-adductoom onder invloed van de vertering van vlees. Wanneer alle *in vitro* en *in vivo* resultaten gebundeld worden, blijkt dat 7 verschillende DNA-adducten een sterke associatie vertonen met de vertering van rood vlees; het betreft O^6-CMG, dimethyl- or ethyl-T, methyl-G, malondialdehyde-2x-G, heptanal-G en malondialdehyde-3x-C (M_3C). Aangezien deze DNA-adducten gevormd worden door de alkylatie en/of oxidatie van DNA door genotoxische verbindingen zoals bv. NOC's en VPO's, zijn deze bevindingen uiterst relevant voor de rood

vlees-DDK hypothese. In lijn met deze resultaten, kon een toename in vetperoxidatie kon meermaals aangetoond worden, hoewel dit niet het geval was voor de vorming van NOC's. Bijgevolg is bijkomend onderzoek noodzakelijk om de rol van de vorming van DNA-adducten in relatie tot de consumptie van rood vlees en de ontwikkeling van DDK volledig op te helderen. Bevestiging van de humane *in vivo* relevantie van de teruggevonden DNA-adducten is daarbij van groot belang. In deze context zou het simultaan gebruik van complementaire omics technieken een grote meerwaarde betekenen. Zo biedt de fusie van metagenomics, metabolomcis en DNA-adductomics erg gunstige toekomstperspectieven, met de belofte van diepgaander fundamenteel (vervolg)onderzoek. Wat betreft het gebruik van DNA-adductomics, moet vermeld worden dat dit onderzoeksveld kampt met 2 belangrijke knelpunten door beperkingen met betrekking tot de beschikbare staalgrootte (hoeveelheid DNA) en de onweerlegbare identificatie van gedetecteerde DNA-adducten. De optimalisatie van (het gebruik van) MS-hybriden en de implementatie van chromatografische innovaties kan hierbij echter soelaas bieden.

Curriculum Vitae

PERSONALIA

Name	Lieselot Yvette Hemeryck
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Date of birth	21th of November, 1988
Place of birth	Oostende
Nationality	Belgian

EDUCATION

Primary education VBS Driespan, Moere (1994-2000)

Secondary education Sint-Godelievecollege, Gistel (2000-2006)

Higher education

Bachelor of Veterinary Medicine (2006-2009)

Magna cum laude

Ghent University, Faculty of Veterinary Medicine, Merelbeke (2006-2009)

Master of Veterinary Medicine (2009-2012)

Magna cum laude

Ghent University, Faculty of Veterinary Medicine, Merelbeke (2009-2012)

Master Thesis entitled "Endogenous formation of thyreostats in livestock"

Doctoral schools

Transferable skills

a) Teaching assistant training (24th of June, 2013, Gent, Belgium)
b) Speed Reading (2nd of December, 2013, Gent, Belgium)
c) Personal Effectiveness (2nd, 9th & 16th of December 2014, Gent, Belgium)

Specialist courses

a) Elementary statistics for researchers; basic course & regression analysis (November-March, 2013-2014)
b) From science to treatment innovation in cancer (11th & 12th of September, 2014, Brussel, Belgium)
c) Clinical studies: study design, implementation and reporting (17th & 18th of September, 2015, Gent, Belgium)

Additional courses & workshops

a) Laboratory Animal Course (2011-2012, Gent, Belgium)
b) 'Future challenges and changes in the meat inspection system in the EU' study evening (19th of September, 2012, Merelbeke, Belgium)
c) TraceFinder 3.0 Software training (9th of April, 2013, Breda, The Netherlands)
d) Hands-on Q-Exactive training (18th & 19th of June, 2014, Breda, The Netherlands)
e) Q-Exactive software training; TraceFinder & Sieve (3rd & 4th of June, 2015, Merelbeke, Belgium)
f) Workshop Feedback (24th of September, 2015, Gent, Belgium)
g) Writing for non-peers and press (11th & 18th of January, Gent, Belgium)

PROFESSIONAL ACTIVITIES

1. **Teaching Assistant** (2012-2017):
   a) Practical sessions 'Analytical Chemistry' (in 1st Bachelor of Veterinary Medicine)
   b) Practical sessions 'Food and Environmental Chemistry' (in 2nd Bachelor of Veterinary Medicine)
   c) Workshops 'Applied Biomedical Techniques' (in 3rd Master of Veterinary Medicine; option Research)

2. **PhD Student** (2012-2017): "Investigation of DNA adduct formation due to the gastrointestinal digestion of red meat to further unravel the causal link between red meat digestion and colorectal cancer"

3. **Food Biomarkers Alliance consortium member** (FOODBALL, JPI HDHL)

4. **Project support & follow-up**
    a) FOD Research Project (RF 11/6250) "MEATNOX": "Formation and (geno)toxic activity of nitros(yl)ation and oxidation products in the gastrointestinal tract: role of nitrate-cured meat products and prevention".
    b) FOD Research Project "ZENDONCONVERT" (RF 14/09): "Investigation on the conversion profile of masked deoxynivalenol and zearalenone in the porcine and human gastrointestinal system".
    c) FOD Research Project "DOSERESIST" (RF 14/6287): "Influence of dosage, administration route and intestinal health on antibacterial resistance selection in intestinal commensal flora of pigs and optimization of dosage regimen of selected antibacterial drugs".
    d) IWT Grant for Strategic Basic Research: "An integrated approach based on metabolomic and tocicological profiling to unravel the link between red meat digestion and colon cancer".
    e) FWO Research Project (G011615N): "Red and processed meat in the diet: toxicological and metabolomic profiling under simulated gastrointestinal conditions".
    f) Independent Research Project: "Advanced metabolomics to predict the course of Crohn's disease", in collaboration with Prof. M. De Vos from UZ Gent (Belgium).
    g) Independent Research Project: "Phenotyping of pediatric patients with Hirschsprung's Disease via the fecal metabolome", in collaboration with Prof. M. Van Winckel from UZ Gent (Belgium).

STUDENTS

1. **Literature studies**
    a) Kristien Van Hoof (2013-2014): "The role of nutrition in humane colorectal cancer pathogenesis".
    b) Olivier Courtens (2013-2014): "The influence of nutrition on the induction and/or inhibition of colorectal cancer in humans".
    c) Anneleen Michielsen (2013-2014): "Biomarkers in colon cancer research".
    d) Lies Claeys (2014-2015): "The role of gut microbiota in health and disease".
    e) Helena Berlamont (2014-2015): "The importance of DNA adducts in humane diet-related cancers".

f) Ine Vermeulen (2015-2016): "Diet and lifestyle related DNA damage".

g) Philip Joosten (2015-2016): "The potential role of DNA adducts in the molecular pathways that lead to colon carcinogenesis".

2. Bachelor and Master projects

a) Jochen Duyck (Master Industrial Engineering, 2012-2013): "Analysis of O^6-carboxymethylguanine and malondialdehyde as markers for an elevated colorectal cancer risk during *in vitro* digestion of meat"

b) Ellen Ooms (Bachelor Biochemistry, 2013-2014): "UHPLC-HRMS analysis of DNA adducts".

c) Caroline Rombouts (Master of Veterinary Medicine, 2013-2014): "Mechanistic investigation of the role of the gastrointestinal digestion of meat and meat products in the pathogenesis of colorectal cancer".

d) Olivier Courtens (Master of Veterinary Medicine, 2014-2015): "Formation of DNA adducts in the colon: the interfering role of diet and individual".

e) Simon Bos (Master of Science in Pharmaceutical Care, 2014-2015): "Metabolomics and DNA adductomics method optimization to study the relation between red meat and colorectal cancer by means of the HT29 cell line".

f) Davy Van Herrewege (Master of Veterinary Medicine, 2016-2017): "Metabolomics for the follow-up of pediatric Hirschrsprung's disease patients after chirurgical intervention."

g) Jitse Loyens (Master of Veterinary Medicine, 2017-2019): "Disruption of the gastro-intestinal metabolome of children suffering chronic bowel diseases."

3. Member of the reading and examination committee of several bachelor and master projects.

PUBLICATIONS

1. Journal articles (A1)

Kiebooms JA, Vanden Bussche J, **Hemeryck LY**, Fievez V, Vanhaecke L. Intestinal microbiota contribute to the endogenous formation of thiouracil in livestock. J Agric Food Chem. 2012 Aug 15;60(32):7769-76 (IF 2012 = 3.334, ranking = 10/124 in Food Science and Technology).

Vanden Bussche J*, **Hemeryck LY***, Van Hecke T, Kuhnle GG, Pasmans F, Moore SA, Van de Wiele T, De Smet S, Vanhaecke L. O^6-carboxymethylguanine DNA adduct formation and lipid peroxidation upon *in vitro* gastrointestinal digestion of haem-rich meat. Mol Nutr Food Res. 2014 Sep;58(9):1883-96 (IF 2014 = 4.603, ranking = 4/123 in Food Science and Technology). *shared first author

Van Hoof KJM, **Hemeryck LY**, Vanhaecke L. Consumptie van rood en verwerkt vlees en humane colorectale kanker: is er een verband? Vlaams Diergeneeskundig Tijdschrift. 2015 Jan-Feb;84(1):3-9 (IF 2015 = 0.203, ranking = 124/138 in Veterinary Sciences).

Van Hecke T, Vossen E, **Hemeryck LY**, Vanden Bussche J, Vanhaecke L, De Smet S. Increased oxidative and nitrosative reactions during digestion could contribute to the association between well-done red meat consumption and colorectal cancer. Food Chem. 2015 Nov 15;187:29-36 (IF 2015 = 4.052, ranking = 7/125 in Food Science and Technology).

Hemeryck LY, Decloedt AI, Vanden Bussche J, Geboes KP, Vanhaecke L. High resolution mass spectrometry based profiling of diet-related deoxyribonucleic acid adducts. Anal Chim Acta. 2015 Sep 10;892:123-31 (IF 2015 = 4.712, ranking = 8/75 in Chemistry, Analytical).

Hemeryck LY, Vanhaecke L. Diet-related DNA adduct formation in relation to carcinogenesis. Nutr Rev. 2016 Aug;74(8):475-89 (IF 2015 = 5.591, ranking = 4/80 in Nutrition & Dietetics).

Hemeryck LY, Moore SA, Vanhaecke L. Mass Spectrometric Mapping of the DNA Adductome as a Means to Study Genotoxin Exposure, Metabolism, and Effect. Anal Chem. 2016 Aug 2;88(15):7436-46 (IF 2015 = 5.886, ranking = 4/75 in Chemistry, Analytical).

Hemeryck LY, Rombouts C, Van Hecke T, Van Meulebroek L, Vanden Bussche J, De Smet S, Lynn Vanhaecke L. *In vitro* DNA adduct profiling to mechanistically link red meat consumption to colon cancer promotion. Toxicol Res. 2016 Sep 1;5:1346-58 (IF 2015 = 2.161, ranking = 51/90 in Toxicology).

Rombouts C, **Hemeryck LY**, Van Hecke T, De Smet S, De Vos W, Vanhaecke L. Untargeted metabolomics of colonic digests reveals kynurenine pathway metabolites, dityrosine and 3-

dehydroxycarnitine as red versus white meat discriminating metabolites. Sci Rep. 2017, Feb 14;7:42514 (IF 2015 = 5.228, ranking = 7/63 in Multidisciplinary Sciences).

Hemeryck LY, Van Hecke T, Vossen E, De Smet S, Vanhaecke L. DNA adductomics to study the genotoxic effects of red meat consumption with and without added animal fat in rats. Food Chem. 2017, Sep 1;230:378-387 (IF 2015 = 4.052, ranking = 7/125 in Food Science and Technology).

Hemeryck LY, Rombouts C, De Paepe E, Vanhaecke L. DNA adduct profiling of *in vitro* colonic meat digests to map red vs. white meat genotoxicity. 2017, *submitted*.

Praticó G, **Hemeryck LY**, Pedapati SH, Cuparencu CS, Rombouts C, Vanhaecke L, Hanhineva K, Brennan L, Dragsted LO. Intake biomarkers for food of animal origin: biomarkers of meat intake. Genes Nutr. 2017, *in preparation*.

2. Proceedings (conferences & symposia)

Hemeryck LY, Vanden Bussche J, Moore S, De Smet S, Vanhaecke L. Does digestion of red meat cause more procarcinogenic DNA adducts in healthy subjects as opposed to white meat? 59th International Congress of Meat Science and Technology (ICoMST) (p. 14–17), 2013, **oral presentation**, Izmir, Turkey.

Rombouts C, **Hemeryck LY**, Van Hecke T, Vanden Bussche J, De Smet S, Vanhaecke L. High-resolution mass spectrometry based metabolomics reveals acylcarnitines as key metabolites discriminating red from white meat colonic digestion. 61st International Congress of Meat Science and Technology (ICoMST) (p. 256), 2013, oral presentation by Rombouts C, Clermont-Ferrand, France.

3. Abstracts (conferences & symposia)

Vanden Bussche J, **Hemeryck LY**, Van Hecke T, De Smet S, Vanhaecke L. Do gastrointestinal bacteria affect the (geno)toxic activity of different meats? 14th Gut Day Symposium, 2012, poster presentation by Vanden Bussche J, Leuven, Belgium.

Hemeryck LY, Vanden Bussche J, Vanhaecke L. U-HPLC coupled to high resolution Orbitrap mass spectrometry to screen for colonic DNA adduct formation following meat consumption. Trends in Food Analysis VII (KVCV), 2013, **poster presentation**, Gent, Belgium.

Hemeryck LY, Vanden Bussche J, Van Hecke T, De Smet S, Vanhaecke L. Does the healthy human colonic microbiota give rise to the formation of cyto- and genotoxic compounds upon red meat digestion? 15th Gut Day Symposium, 2013, **poster presentation**, Groningen, The Netherlands.

Hemeryck LY, Vanden Bussche J, Van Hecke T, De Smet S, Vanhaecke L. Does *in vitro* gastrointestinal digestion of red meat, in comparison to white meat, give rise to the formation of more cyto- and genotoxic compounds? BelTox Annual Scientific Meeting, 2013, **oral presentation**, Louvain-la-Neuve, Belgium.

Hemeryck LY, Vanden Bussche J, Vanhaecke L. Screening for DNA adducts in meat digests with UHPLC coupled to high-resolution Orbitrap mass spectrometry in colorectal cancer research. 13th International symposium on Hyphenated Techniques in Chromatography and Separation Technology (HTC-13), 2014, **oral presentation**, Brugge, Belgium.

Hemeryck LY, Vanden Bussche J, Vanhaecke L. *In vitro* and *in vivo* screening for diet-related DNA adducts using ultra-high performance liquid chromatography coupled to mass spectrometry. 2nd OncoPoint Meeting, 2014, **short oral communication**, Gent, Belgium.

Hemeryck LY, Rombouts C, Vanden Bussche J, Vanhaecke L. Linking meat consumption to colorectal cancer biomarkers: a metabolomic *in vitro* approach. The Herrenhausen Conference: Beyond the intestinal microbiome : from signatures to therapy, Volkswagenstiftung, 2014, **short oral communication & poster presentation**, Hannover, Germany.

Hemeryck LY, Vanden Bussche J, Vanhaecke L. Assessing the genotoxicity of the human diet by DNA adduct profiling. BelTox Annual Scientific Meeting, 2014, **poster presentation**, Geel, Belgium.

Hemeryck LY, Decloedt A, Vanden Bussche J, Geboes K, Vanhaecke L. *In vitro* and *in vivo* diet related DNA adduct profiling in search of a colon cancer biomarker. 3rd OncoPoint Meeting, 2015, **short oral communication**, Gent, Belgium.

Hemeryck LY, Rombouts C, Van Meulebroek L, Vanden Bussche J, Vanhaecke L. Mapping of the DNA adductome to study the genotoxic effects of red meat consumption. 12th NuGO Week: Mechanisms of a long-life health, 2015, **oral presentation**, Barcelona, Spain.

Hemeryck LY, Rombouts, C, Van Meulebroek L, Vanden Bussche J, Vanhaecke L. DNA adductomics to unravel the genotoxic effects of red meat consumption. 2nd Symposium of Mass Spectrometry in Food and Feed, 2015, **oral presentation**, Gent, Belgium.

Hemeryck LY, Rombouts C, Van Hecke T, De Smet S, Vanden Bussche J, De Vos W, Vanhaecke L. High resolution mass spectrometry based digestion metabolomics and DNA adductomics reveal red meat digestion associated biomarkers. Belgian Association for Meat Science and Technology Symposium (BAMST), 2015, short oral communication & poster presentation by Vanhaecke L, Melle, Belgium.

Vanden Bussche J, **Hemeryck LY**, Rombouts C, Van Meulebroek, L, Vanhaecke L. Digestion metabolomics and DNA adductomics as a tool for phenotype discrimination. MetaboMeeting 2015, 2015, poster presentation by Vanden Bussche J, Cambridge, United Kingdom.

Vanhaecke L, **Hemeryck LY**, Rombouts C, Van Meulebroek L, Vanden Bussche J UHPLC coupled to hybrid high resolution MS for digestion metabolomics and DNA adductomics. 14th International symposium on Hyphenated Techniques in Chromatography and Separation Technology (HTC-14), 2016, oral presentation by Vanhaecke L, Gent, Belgium.

Praticó G, Manach C, Kolehmainen M, Garcia-Aloy M, Brennan L, Scalbert A, Afman L, Vazquez-Fresno R, **Hemeryck LY**, Münger L, Hanhineva K, Dragsted LO New classification and validation system for intake biomarkers to improve assessment of food intake and nutritional status – A FoodBAll project goal. 12th Annual Conference of the Metabolomics Society, 2016, oral presentation by Praticó G, Dublin, Ireland.

Hemeryck LY, Van Hecke T, Vossen E, De Smet S, Vanhaecke L. DNA adductomics to reveal red meat and high fat intake related genotoxicity in rats. 13th NuGO week: Phenotypes and prevention – the interplay of genes, life-style and gut environment, 2016, **short oral communication & poster presentation**, Copenhagen, Denmark.

Hemeryck LY, Van Hecke T, Vossen E, De Smet S, Vanhaecke L. Red meat and animal fat intake induce DNA adduct formation in rat liver, duodenum and colon. 18th Gut Day Symposium, 2016, **poster presentation**, Venlo, The Netherlands.

Rombouts C, **Hemeryck LY**, Van Hecke T, De Smet S, De Vos W, Vanhaecke L. Untargeted metabolomics of colonic digests reveals kynurenine pathway metabolites, dityrosine and 3-dehydroxycarnitine as red versus white meat discriminating metabolites. 18th Gut Day Symposium, 2016, oral presentation by Rombouts C, Venlo, The Netherlands.

Rombouts C, **Hemeryck LY**, Van Hecke T, De Smet S, De Vos W, Vanhaecke L. Mass spectrometry based untargeted metabolomics of colonic digests reveals tryptophan catabolites, dityrosine and 3-dehydroxycarnitine as red versus white meat discriminating microbial metabolites. Belgian Association for Meat Science and Technology Symposium (BAMST), 2016, oral presentation by Rombouts C, Melle, Belgium.

AWARDS & GRANTS

1. Prize for the best Master Thesis in Veterinary Medicine, option Research, class of 2012.
2. Award (First prize) for an Outstanding Scientific Communication at the 'BELTOX Annual Meeting' at Louvain-La-Neuve, 6th of December 2013.
3. FWO Grant for participation in a conference abroad (NuGO week in Copenhagen (Denmark), 5 – 9 September 2016).

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Thank you very muchers! #mylove #kisskiss

Evert, onze toekomst lijkt alvast bijzonder rooskleurig. En hoewel ik er niet bepaald naar uitkijk om 's morgensvroeg of middenin de nacht op te staan, begin ik meer en meer te verlangen naar ons volgend projectje, a.k.a. 'Jefke'. Ik hoop alvast dat hij op zijn papa lijkt :-). #mybabyboy

Lieselot

Merelbeke, 15 mei 2017